## Encoded Molecules by Translation (EMT)

### Technical Field of the Invention

5    The present invention relates to the field of polymers and branched molecules encoded by a template, i.e. templated molecules. Furthermore, the invention relates to methods of preparing encoded polymers and encoded branched molecules.

### Background

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One central dogma in biology describes the flow of information as a one-way process from DNA to RNA to polypeptide. DNA is transcribed by a RNA polymerase into mRNA; and the mRNA is subsequently then translated into protein by the ribosomes and tRNAs.

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The direct relation between DNA and protein, i.e., the fact that the sequence of triplet codons defines the sequence of α-amino acid residues in a polypeptide, has allowed the development of numerous molecular biological methods, wherein DNA may be manipulated (mutagenizes, recombines, deletes, inserts, etc), and then

20    used in in vivo systems (e.g., microbes) or in vitro systems (e.g., Zubay in vitro expression systems) to transfer the resulting changes from DNA level to the level of templated polypeptide, i.e., to mutate, recombine, delete, insert, etc. the polypeptide.

25    Several systems have been invented that allows a flow of information from polypeptide to DNA the so-called retro-genetics. These systems include phage display, ribosome/polysome display, peptides-on-plasmid display, and other systems. These systems introduce a physical link between the template (e.g., DNA or RNA) and the templated polypeptide. As a result, it is possible, from a population

30    of templated polypeptides linked to their respective templates, to first enrich for a desired characteristic of the templated polypeptide (e.g., binding of the templated molecule to an affinity column), and subsequently amplify the enriched population of templated polypeptides through amplification of its template (DNA or RNA), followed by translation of the amplified templates. These methods have been used to identify

polypeptides with novel and/or improved features from libraries that may consist of more than $10^{14}$ polypeptides.

The critical feature of the prior art systems is the amplifiability of the templated molecule, through amplification of its template. Thus, after the selection step in which molecules with the desired property are enriched, the enriched population may be amplified and then taken through yet a selection step, etc. - the process of selection-and-amplification may be repeated many times. In this way the "noise" of the selection assay is averaged out over several selection-and-amplification rounds, and even if the individual selection step yield only modest enrichment e.g. 10-fold, a theoretical enrichment of $10^{12}$ can be reached after 12 selection-and-amplification rounds.

In the field of chemistry, a different combinatorial approach has been developed. This approach involved the parallel synthesis of millions of related compounds, in an array (where each position defined a specific compound), or on beads (where one bead carried many copies of the same compound). The population of compounds are then screened for desired characteristics. Importantly, this type of combinatorial library has no means for amplification, and therefore requires the use of very stringent screening methods, as explained above.

Principles for tagging chemical libraries have also been developed. For example, systems that employed DNA oligos to tag molecule libraries have been developed as exemplified herein below. The tag is used as a means of identification, but cannot be used to template the synthesis of the tagged molecule. Therefore, despite the tag, these systems still require a very efficient screening method.

Below prior art in the field of the invention is summarised:

WO 00/23458 describes combinatorial libraries, wherein each library component comprises a nucleic acid tag, which encodes the synthesis of a polymeric compound covalently attached thereto. The synthesis of the compound requires a "split and recombine" strategy. Hence, the synthesis can not be performed in one closed chamber, but requires that the library components are split according to the

sequence of their respective nucleic acid tags, before addition of a unit to the polymeric compound.

EP 0 604 552 B1 relates to a method for synthesizing diverse collections of oligomers. The invention involves the use of an identifier tag to identify the sequence of monomers in an oligomer. The identifier tags facilitate subsequent identification of reactions through which members of a library of different synthetic compounds have been synthesised in a component by component fashion.

EP 0 643 778 B1 relates to encoded combinatorial chemical libraries. Each of a collection of polypeptides is labelled by an appended "genetic" tag, itself constructed by chemical synthesis, to provide a "retro-genetic" way of specifying each polypeptide.

EP 0 773 227 A1 relates to a method for preparing a new pharmaceutical drug or diagnostic reagent, which includes the step of screening, against a ligand or receptor, a library of different synthetic compounds obtainable by synthesis in a component by component fashion.

US 4,863,857 relates to a method for determining the amino acid sequence of a polypeptide complementary to at least a portion of an original peptide or protein. In one aspect the method involves: (a) determining a first nucleotide sequence of a first nucleic acid coding for the biosynthesis of at least a portion of the original peptide or protein; (b) ascertaining a second nucleotide sequence of a second nucleic acid which base-pairs with the first nucleotide sequence of the first nucleic acid, the first and second nucleic acids pairing in antiparallel directions; and (c) determining the amino acid sequence of the complementary polypeptide by the second nucleotide sequence when read in the same reading frame as the first nucleotide sequence.

US 5,162,218 relates to polypeptide compositions having a binding site specific for a particular target ligand and further having an active functionality proximate the binding site. The active functionality may be a reporter molecule, in which case the polypeptide compositions are useful in performing assays for the target ligand. Also disclosed are methods for preparing polypeptides having active functionalities proximate their binding site, said method comprising the step of combining the

polypeptide specific for the target ligand with an affinity label having a reactive group attached thereto. The reactive group is then covalently attached to an amino acid side chain proximate the binding site and cleaved from the substrate. The substrate is subsequently eluted, leaving a moiety of the reactive group covalently attached to the polypeptide. The active functionality may then be attached to the moiety.

US 5,270,170 relates to a random peptide library constructed by transforming host cells with a collection of recombinant vectors that encode a fusion protein comprised of a DNA binding protein and a random peptide and also encode a binding site for the DNA binding protein. The fusion protein can be used for screening ligands. The screening method results in the formation of a complex comprising the fusion protein bound to a receptor through the random peptide ligand and to the recombinant DNA vector through the DNA binding protein.

US 5,574,141 relates to functionalized carrier materials for the simultaneous synthesis and direct labelling of oligonucleotides as primers for template-dependent enzymatic nucleic acid syntheses. The polymeric carriers are loaded with nucleic acid building blocks which in turn contain labelling groups or precursors thereof. The polymeric carrier loaded in this way serves as a solid or liquid phase for the assembly of oligonucleotides which can be used as primers for a template-dependent enzymatic nucleic acid synthesis such as in sequencing analysis or in the polymerase chain reaction (PCR).

US 5,573,905 relates to an encoded combinatorial chemical library comprising a plurality of bifunctional molecules having both a chemical polymer and an identifier oligonucleotide sequence that defines the structure of the chemical polymer. Also described are the bifunctional molecules of the library, and methods of using the library to identify chemical structures within the library that bind to biologically active molecules in pre-selected binding interactions.

US 5,597,697 relates to a screening assay for inhibitors and activators of RNA and DNA-dependent nucleic acid polymerases. The invention provides methods for the identification and discovery of agents which are inhibitors and activators of RNA and DNA-dependent nucleic acid polymerases. The essential feature of the invention is the incorporation of a functional polymerase binding site sequence (PBS) into a

nucleic acid molecule which is chosen for its ability to confer a discernible characteristic via its sequence specific activity such that the incorporation of the PBS renders the nucleic acid molecule a functional template for a predetermined RNA or DNA-template directed nucleic acid polymerase. In the presence of the polymerase, suitable primer molecules, and any necessary accessory molecules, catalytic extension of the strand of nucleic acids complementary to the template occurs, resulting in a partial or total elimination of (or increase in) the characteristic conferring activity of the reporter-template molecule described due to the antisense effects of the complementary strand or other polymerase-mediated effects.

US 5,639,603 relates to a method for synthesizing and screening molecular diversity by means of a general stochastic method for synthesizing compounds. The method can be used to generate large collections of tagged compounds that can be screened to identify and isolate compounds with useful properties.

US 5,698,685 relates to a morpholino-subunit combinatorial library and a method for generating a compound capable of interacting specifically with a selected macromolecular ligand. The method involves contacting the ligand with a combinatorial library of oligomers composed of morpholino subunits with a variety of nucleobase and non-nucleobase side chains. Oligomer molecules that bind specifically to the receptor are isolated and their sequence of base moieties is determined. Also disclosed is a combinatorial library of oligomers useful in the method and novel morpholino-subunit polymer compositions.

US 5,708,153 relates to a method for synthesizing diverse collections of tagged compounds by means of a general stochastic method for synthesizing random oligomers on particles. A further aspect of the invention relates to the use of identification tags on the particles to facilitate identification of the sequence of the monomers in the oligomer.

US 5,719,262 relates to a novel class of compounds, known as peptide nucleic acids, which bind complementary DNA and RNA strands more strongly than the corresponding DNA or RNA strands, and exhibit increased sequence specificity and solubility. The peptide nucleic acids comprise ligands selected from a group consisting of naturally-occurring nucleobases and non-naturally-occurring

nucleobases attached to a polyamide backbone, and contain alkylamine side chains.

US 5,721,099 relates to encoded combinatorial chemical libraries encoded with tags. Encoded combinatorial chemistry is provided, whereby sequential synthetic schemes are recorded using organic molecules, which define choice of reactant, and stage, as the same or different bit of information. Various products can be produced in the multi-stage synthesis, such as oligomers and synthetic non-repetitive organic molecules. Particularly, pluralities of identifiers may be used to provide a binary or higher code, so as to define a plurality of choices with only a few detachable tags. The particles may be screened for a characteristic of interest, particularly binding affinity, where the products may be detached from the particle or retained on the particle. The reaction history of the particles which are positive for the characteristic can be determined by the release of the tags and analysis to define the reaction history of the particle.

US 5,723,598 relates to an encoded combinatorial chemical library comprised of a plurality of bifunctional molecules having both a chemical polymer and an identifier oligonucleotide sequence that defines the structure of the chemical polymer. Also described are the bifunctional molecules of the library, and methods of using the library to identify chemical structures within the library that bind to biologically active molecules in preselected binding interactions.

US 5,770,358 relates to tagged synthetic oligomer libraries and a general stochastic method for synthesizing random oligomers. The method can be used to synthesize compounds to screen for desired properties. The use of identification tags on the oligomers facilitates identification of oligomers with desired properties.

US 5,786,461 relates to peptide nucleic acids having amino acid side chains. A novel class of compounds, known as peptide nucleic acids, bind complementary DNA and RNA strands more strongly than the corresponding DNA or RNA strands, and exhibit increased sequence specificity and solubility. The peptide nucleic acids comprise ligands selected from a group consisting of naturally-occurring nucleobases and non-naturally-occurring nucleobases attached to a polyamide backbone, and contain alkylamine side chains.

US 5,789,162 relates to a method for synthesizing diverse collections of oligomers. A general stochastic method for synthesizing random oligomers on particles is disclosed. A further aspect of the invention relates to the use of identification tags on the particles to facilitate identification of the sequence of the monomers in the oligomer.

US 5,840,485 relates to topologically segregated, encoded solid phase libraries. Libraries of synthetic test compounds are attached to separate phase synthesis supports that also contain coding molecules that encode the structure of the synthetic test compound. The molecules may be polymers or multiple nonpolymeric molecules. The synthetic test compound can have backbone structures with linkages such as amide, urea, carbamate (i.e., urethane), ester, amino, sulfide, disulfide, or carbon-carbon, such as alkane and alkene, or any combination thereof. The synthetic test compound can also be molecular scaffolds, or other structures capable of acting as a scaffold. The invention also relates to methods of synthesizing such libraries and the use of such libraries to identify and characterize molecules of interest from among the library of synthetic test compounds.

US 5,843,701 relates to systematic polypeptide evolution by reverse translation and a method for preparing polypeptide ligands of target molecules wherein candidate mixtures comprised of ribosome complexes or mRNA:polypeptide copolymers are partitioned relative to their affinity to the target and amplified to create a new candidate mixture enriched in ribosome complexes or mRNA:polypeptide copolymers with an affinity to the target.

US 5,846,839 relates to a method for hard-tagging an encoded synthetic library. Disclosed are chemical encryption methods for determining the structure of compounds formed in situ on solid supports by the use of specific amine tags which, after compound synthesis, can be deencrypted to provide the structure of the compound found on the support.

US 5,922,545 relates to methods and compositions for identifying peptides and single-chain antibodies that bind to predetermined receptors or epitopes. Such

peptides and antibodies are identified by methods for affinity screening of polysomes displaying nascent peptides.

US 5,958,703 relates to methods for screening libraries of complexes for compounds having a desired property such as the capacity to bind to a cellular receptor. The complexes in such libraries comprise a compound under test, a tag recording at least one step in synthesis of the compound, and a tether susceptible to modification by a reporter molecule. Modification of the tether is used to signify that a complex contains a compound having a desired property. The tag can be decoded to reveal at least one step in the synthesis of such a compound

US 5,986,053 relates peptide nucleic acid complexes of two peptide nucleic acid strands and one nucleic acid strand. Peptide nucleic acids and analogues of peptide nucleic acids are used to form duplex, triplex, and other structures with nucleic acids and to modify nucleic acids. The peptide nucleic acids and analogues thereof also are used to modulate protein activity through, for example, transcription arrest, transcription initiation, and site specific cleavage of nucleic acids.

US 5,998,140 relates to methods and compositions for forming complexes intracellularly between dsDNA and oligomers of heterocycles, aliphatic amino acids, particularly omega-amino acids, and a polar end group. By appropriate choice of target sequences and composition of the oligomers, complexes are obtained with low dissociation constants.

US 6,060,596 relates to an an encoded combinatorial chemical library comprised of a plurality of bifunctional molecules having both a chemical polymer and an identifier oligonucleotide sequence that defines the structure of the chemical polymer. Also described are the bifunctional molecules of the library, and methods of using the library to identify chemical structures within the library that bind to biologically active molecules in preselected binding interactions.

US 6,080,826 relates to Template-directed ring-closing metathesis and ring-opening metathesis polymerization of functionalized dienes. Functionalized cyclic olefins and methods for making the same are disclosed. Methods include template-directed ring-closing metathesis ("RCM") of functionalized acyclic dienes and template-

directed depolymerization of functionalized polymers possessing regularly spaced sites of unsaturation. Although the template species may be any anion, cation, or dipolar compound, cationic species, especially alkali metals, are preferred. Functionalized polymers with regularly spaced sites of unsaturation and methods for

5    making the same are also disclosed. One method for synthesizing these polymers is by ring-opening metathesis polymerization ("ROMP") of functionalized cyclic olefins.

US 6,127,154 relates to compounds which possess a complementary structure to a desired molecule, such as a biomolecule, in particular polymeric or oligomeric

10   compounds, which are useful as in vivo or in vitro diagnostic and therapeutic agents are provided. Also, various methods for producing such compounds are provided.

US 6,140,493 relates to a method for synthesizing diverse collections of oligomers. A general stochastic method for synthesizing random oligomers is disclosed and

15   can be used to synthesize compounds to screen for desired properties. Identification tags on the oligomers facilitates identification of oligomers with desired properties.

US 6,140,496 relates to building blocks for preparing oligonucleotides carrying non-standard nucleobases that can pair with complementary non-standard nucleobases

20   so as to fit the Watson-Crick geometry. The resulting base pair joins a monocyclic six membered ring pairing with a fused bicyclic heterocyclic ring system composed of a five member ring fused with a six member ring, with the orientation of the heterocycles with respect to each other and with respect to the backbone chain analogous to that found in DNA and RNA, but with a pattern of hydrogen bonds

25   holding the base pair together different from that found in the AT and GC base pairs (a "non-standard base pair").

US 6,143,497 relates to a method for synthesizing diverse collections of random oligomers on particles by means of a general stochastic method. Also disclosed are

30   identification tags located on the particles and used to facilitate identification of the sequence of the monomers in the oligomer.

US 6,165,717 relates to a general stochastic method for synthesizing random oligomers on particles. Also disclosed are identification tags located on the particles

35   to facilitate identification of the sequence of the monomers in the oligomer.

US 6,175,001 relates to functionalized pyrimidine nucleosides and nucleotides and DNA's incorporating same. The modified pyrimidine nucleotides are derivatized at C5 to contain a functional group that mimics the property of standard amino acid residues. DNA molecules containing the modified nucleotides are also provided.

US 6,194,550 B1 relates to systematic polypeptide evolution by reverse translation, in particular a method for preparing polypeptide ligands of target molecules wherein candidate mixtures comprised of ribosome complexes or mRNA:polypeptide copolymers are partitioned relative to their affinity to the target and amplified to create a new candidate mixture enriched in ribosome complexes or mRNA:polypeptide copolymers with an affinity to the target.

US 6,207,446 B1 relates to methods and reagents for the selection of protein molecules that make use of RNA-protein fusions.

US 6,214,553 B1 relates to methods and reagents for the selection of protein molecules that make use of RNA-protein fusions.

WO 91/05058 relates to a method for the cell-free synthesis and isolation of novel genes and polypeptides. An expression unit is constructed onto which semi-random nucleotide sequences are attached. The semi-random nucleotide sequences are first transcribed to produce RNA, and then translated under conditions such that polysomes are produced. Polysomes which bind to a substance of interest are then isolated and disrupted; and the released mRNA is recovered. The mRNA is used to construct cDNA which is expressed to produce novel polypeptides.

WO 92/02536 relates to a method for preparing polypeptide ligands of target molecules wherein candidate mixtures comprised of ribosome complexes or mRNA:polypeptide copolymers are partitioned relative to their affinity to the target and amplified to create a new candidate mixture enriched in ribosome complexes or mRNA:polypeptide copolymers with an affinity to the target.

WO 93/03172 relates to a method for preparing polypeptide ligands of target molecules wherein candidate mixtures comprised of ribosome complexes or

mRNA:polypeptide copolymers are partitioned relative to their affinity to the target and amplified to create a new candidate mixture enriched in ribosome complexes or mRNA:polypeptide copolymers with an affinity to the target.

5      WO 93/06121 relates to a general stochastic method for synthesizing random oligomers on particles. Also disclosed are identification tags located on the particles to facilitate identification of the sequence of the monomers in the oligomer.

WO 00/47775 relates to a method for generating RNA-protein fusions involving a
10     high-salt post-translational step.

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Poly-peptide synthesis by ribosomes involves peptidyl transferase activity
20     coordinating the interaction of a carboxy group of an α-amino acid in the ribosomal
P-site and the amino group of an α-amino acid in the A-site forming an amide bond.
Successive additions of α-amino acids results in formation of an α-peptide (protein).
Each amino acid is presented in the ribosome by an amino acid-specific tRNA that
read (decode) the template mRNA. The sequence of an mRNA is "decoded" in
25     increments of three nucleotides (a codon) specifying a single amino-acid.
Consequently, a nucleotide diversity of 4 (G, A, U, C) in RNA and a triple nucleotide
(codon) read-out will produce 64 different codon sequences each corresponding to
an α-amino acid or protein factors necessary for translation termination. In almost all
natural systems only 20 amino acids specified by 61 codons are incorporated into
30     proteins. Though nature only employs 20 standard amino acids many non-standard
amino acids (NS-AA) and pseudo-amino acids have been incorporated into proteins
both in vitro and in vivo (Liu et al 1999). A non-exhaustive list of non-standard amino
acids and pseudo amino acids incorporated into proteins during the process of
translation is shown in figure 3.

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The chemical diversity of amino acid residues provide a valuable tool for the synthesis of a random or semi-random library of peptide molecules using in vitro or in situ translation (and transcription) in extracts of prokaryotic or eukaryotic origin. From such libraries it is possible to select molecules of desired functionality based on the interaction with a target of interest (for example an affinity column). However, when operating large libraries of for example $10^{14}$ different molecules it is practically impossible to select and identify single molecules of desired properties in a single step. In order to isolate specific peptide sequences with relevant characteristics, it is necessary to include a "retro-genetic element" preferably a DNA or RNA sequence, that templates the synthesis of the peptide sequence. This retro-genetic element enable amplification of a nucleotide sequence that encodes a specific peptide sequence having relevant properties. Furthermore, the retro-genetic element allows multiple rounds of selection and amplification of peptide sequences necessary for the isolation of unique peptides from large libraries. Consequently, several procedures have been developed that allows the formation of nucleic acid-peptide complexes formed by either covalent or non-covalent coupling between a peptide and the RNA or DNA that encode said peptide.

**Peptide Display** (SPERT – Systematic Polypeptide Evolution by Reverse Translation) a protocol acknowledged by those skilled in the art, describes peptide synthesis by ribosomes and the formation of ribosome-mRNA-peptide complexes by incomplete peptide synthesis (i.e. ribosome stalling). Ribosome-mRNA-peptide complexes can be partitioned based on peptide functionality allowing enrichment of complexes with desired properties. Subsequent amplification using reverse transcription and the polymerase chain reaction (PCR) of co-selected RNA sequences permits multiple selection and amplification rounds until a pool of peptide products having desired characteristics are obtained (patent no.: US.6,194,550 B1).

**PROFusion**, a protocol acknowledged by those skilled in the art, describes the covalent attachment of a peptide to the 3'-end of the mRNA which encodes said peptide (Roberts and Szostak, 1997). This protocol enables the synthesis of a library of RNA-peptide complexes that can be used for selection and isolation of peptide molecules with desired properties. Subsequently, the protein sequence information is recovered by amplification of the appended mRNA molecule using reverse transcription and PCR or equivalent techniques. The RNA-protein fusions

permit repeated rounds of selection and amplification allowing enrichment of peptides with relevant characteristics (patent no.: US 6,214,553 B1).

**RIDS** (Ribosome Inactivation Display System), is a protocol describing the connection between genotype and phenotype by formation of a stable complex between a ribosome, an mRNA and its translated protein. The coupling is based on the translation of the ricin A chain which enables cis-inactivation of the ribosome. Thus, predetermined sequences for library synthesis by translation are fused to the ricin A gene. Following synthesis of the peptide-ricin A fusion products the ribosome is inactivated forming stable ribosome-mRNA-peptide ternary complexes enabling selection of peptides having desired properties. Following selection, the appended genetic sequence of is amplified by reverse transcription and PCR.

The RNA-fusion product permits repeated rounds of selection and amplification allowing enrichment of peptides with relevant properties (Zhou et al J. Am. Chem. Soc. 2002. in press.

**Covalent Display Technology**, a protocol describing the coupling of a DNA template and the peptide encoded by said DNA template. A DNA template comprising predetermined sequences fused to the *p2A* gene sequence of *E.coli* bacteriophage T2. Following transcription and translation each fusion peptide product produces a covalent attachment between said peptides and the DNA template that specifies the p2A fusion product. Thus, library of peptide sequences fused to their corresponding DNA templates can be used for selection of relevant peptides and subsequent amplification of the appended DNA template by polymerase chain reaction. Multiple rounds of selection and amplification allows for the isolation of peptide sequences with desired properties.

**Summary of the Invention**

A central process in biology is the formation of polypeptides involving ribosome mediated translation of an RNA template (mRNA). This process converts the genetic information encoded by mRNAs into specific sequences of alpha-amino-acids forming the polypeptides (proteins) that perform nearly all biological processes within a living cell.

Hence, biological systems allow template-directed synthesis of alpha-peptides by the process of ribosome mediated translation of a messenger-RNA (mRNA) template. The present invention describes a system that allows template-directed synthesis of other types of polymers and branched molecules in addition to alpha-peptides. Polymers or branched molecules synthesised by template-directed synthesis (see definition herein below)are designated templated molecules throughout the description.

Templated molecules comprise a plurality of functional groups that are linked together forming a polymer or a branched molecule. Each functional group is usually initially linked to an amino acid or amino acid-like entity (non-standard amino acid or pseudo amino acid) and thus constitutes a non-standard sidegroup of said amino acid or amino acid-like entity. Upon translation of a predetermined mRNA template the incorporation of amino-acids carrying non-standard sidegroups produce peptides carrying appended functional entities. The appended functional entities can subsequently be linked to adjacent functional entities resulting in the formation of a templated molecule linked to its template.

The present invention describes methods to synthesise, select, amplify and isolate templated molecules of desired properties. In particular, the invention overcomes some of the inherent limitations of the technologies described in the prior art, thereby allowing for the synthesis of a variety of differenttemplated molecules that are not limited to α-peptides or modified α-peptides. The present invention enables the synthesis of templated molecules such as β -, γ -, ω peptides, carbopeptoids, vinylogous peptides, oligoanthrilamides, oligoureas, azapeptides (azatides), oligocarbamates, PNA, oligopyrrolinones, vinylogous sulfonamidopeptide, peptoids, azapeptoids or hydrazino peptides. Furthermore it is possible to synthesise templated molecules comprising heterocyclic components for example coumarins and quinolones, pyrazolone, isoxazolone, pyrimidiones, phthalhydrazides, diketopiperazines, hydantoins and benzodiazepines.

Thus, the invention relates to a method for templating molecules. The invention also relates to a method for covalent or non-covalent coupling between a template and templated molecule that in preferred embodiments enable amplification of the templated molecule by amplification of the template encoding it.

The system combines the advantages of the natural system (information flow from template to templated molecule), as well as the recently invented ribosome-mediated systems (e.g., PROFusion), namely the physical link between template and the templated molecule.

Accordingly, it is a first objective of the present invention to provide methods for synthesising a templated molecule comprising a plurality of functional groups, said method comprising the steps of

i)    providing at least one template comprising a sequence of n coding elements selected from the group consisting of first coding elements and second coding elements,

wherein each coding element comprises at least one recognition group capable of recognising a predetermined complementing element, and

wherein n is an integer of at least 3,

with the proviso that the template comprises at least 3 first coding elements,

ii)   providing a plurality of building blocks selected from the group consisting of first building blocks and second building blocks, with the proviso that at least 3 first building blocks are provided,

wherein each first building block comprises

a)  at least one complementing entity comprising a first complementing element comprising at least one recognition group capable of recognising a predetermined first coding element,

b)  at least one functional entity comprising at least one functional group and at least one functional entity reactive group, and

c) at least one spacer comprising at least one spacer reactive group, wherein the spacer is separating the at least one functional entity from the at least one complementing entity, and

wherein each second building block comprises

a) at least one complementing entity comprising a second complementing element comprising at least one recognition group capable of recognising a predetermined second coding element,

b) and at least one spacer comprising at least one spacer reactive group,

complementing coding elements by contacting each coding element with a building block complementing element capable of recognising said coding element, wherein at least 2 coding elements are interacting with complementing elements simultaneously,

with the proviso that a total of at least 3 first coding elements are complemented; and

forming a spacer backbone by linking neighbouring spacers in a ribosome catalysed reaction, by means of reacting spacer reactive groups, and

obtaining a templated molecule comprising at least 3 covalently linked, functional groups by linking, by means of reacting functional entity reactive groups, a functional group of one functional entity to a functional group of another adjacently positioned functional entity and linking said other functional entity to yet another adjacently positioned functional entity.

In one embodiment the present invention relates to the formation of branched molecules comprising a scaffold capable of forming at least 3 covalent bonds to functional groups by means of reactions between reactive groups on the scaffold

and reactive groups of at least 3 functional entities. Formation of branched molecules enables the formation of;

1) Mono, di, tri and oligo functional open-chain hydrocarbons.

2) Mono, di, tri and oligofunctional non-aromatic carbocycles. Monocyclic, bicyclic, tricyclic and polycyclic hydrocarbons. Bridged polycyclic hydrocarbons.

3) Mono, di, tri, and oligofunctional non-aromatic heterocycles. Mono, bi, tri and polycyclic heterocycles. Bridged polycyclic heterocycles.

4) Mono, di, tri and oligo functional aromatic carcocycles. Mono, bi, triand polycyclic aromatic carbocycles.

5) Mono, di, tri and oligofunctional aromatic heterocycles. Mono, bi, tri and polycyclic heterocycles

6) Chelates.

7) Fullerenes

8) Any combination of the above.

It is a second objective of the present invention to provide templated molecules covalently linked to the template encoding said templated molecule, wherein said templated molecule comprises at least 3 covalently linked functional groups, each encoded by a coding element of said template, with the proviso, that the templated molecule is not an α-polypeptide.

It is a third objective of the present invention to provide a plurality of templated molecules, wherein the plurality comprises at least 1000 different templated molecules and wherein said templated molecule comprises at least 3 covalently linked, functional groups, each encoded by a coding element of a template, with the proviso, that the templated molecule is not an α-polypeptide.

**Definitions**

<u>Activation</u>

Activation of a templated molecule involves cleaving one or more of the cleavable linkers that connect functional entities to the spacer backbone.

Adjacently positioned

The adjacently positioned functional entities of any given functional entity are the
functional entities, which are either closest to said given functional entity in 3
dimensional space or which are most likely to react chemically with the given
functional entity.

Preferably, adjacently positioned functional entities of any given functional entity are
the functional entities, which are most likely to react with said functional entity. Often
this will be the functional entity that are closest to said functional entity in 3
dimensional space. Preferably the distance between 2 adjacently positioned
functional entities is on average less than 50 Å, even more preferably less than 20 Å
and most preferably less than 10 Å.

In one preferred embodiment adjacently positioned refers to two functional entities
that are neighbouring on the spacer backbone.

Amino acid residue

The term "amino acid residue" is meant to encompass amino acids, either standard
amino acids, non-standard amino acids or pseudo-amino acids, which have been
reacted with at least one other species, such as 2, for example 3, such as more than
3 other species.

In particular amino acid residues may comprise an acyl bond in place of a free
carboxyl group and/or an amine-bond and/or amide bond in place of a free amine
group. Furthermore, reacted amino acids residues may comprise an ester or
thioester bond in place of an amide bond

AmplificationAmplification according to the present invention is the process wherein
a plurality of exact copies of a starting molecule is synthesised, without employing
knowledge of the exact composition of the starting molecule. Hence a template may
be amplified even though the exact composition of said template is unknown.

In one preferred embodiment of the present invention amplification of a template comprises the process wherein a template is copied by a nucleic acid polymerase or polymerase homologue, for example a DNA polymerase or an RNA polymerase. For example, templates may be amplified using reverse transcription, the polymerase chain reaction (PCR), ligase chain reaction (LCR), in vivo amplification of cloned DNA, and similar procedures capable of complementing a nucleic acid sequence.

Anticodon

An anticodon is a sequence of 3 ribonucleotides that can pair with the bases of a corresponding codon on a messenger RNA.

In certain aspects of the invention it may be favourable to design anticodons that comprise more than 3 nucleotides, such as 4 ribonucleotides, such as 5 ribonucleotides, such as 6 ribonucleotides, such as more than 6 ribonucleotides, for example 8 ribonucleotides or for example 10 ribonucleotides.

Building block

Building blocks according to the present invention may be selected from the group consisting of first building blocks and second building blocks.

A first building block according to the present invention comprises:

at least one complementing entity comprising a first complementing element comprising at least one recognition group capable of recognising a predetermined first coding element,

at least one functional entity comprising at least one functional group and at least one functional entity reactive group, and

at least one spacer comprising at least one spacer reactive group, wherein the spacer is separating the at least one functional entity from the at least one complementing entity.

Preferably, the functional entity is separated from the spacer by a cleavable linker.

A specific example of a first building block is depicted in figure 4A.

5    A second building block according to the present invention comprises:

at least one complementing entity comprising a second complementing element comprising at least one recognition group capable of recognising a predetermined second coding element,

10

and at least one spacer comprising at least one spacer reactive group.

A specific example of a second building block is depicted in figure 4B.

15    <u>Charging</u>

Charging is the step in the synthesis of a first or a second building block, in which a spacer is coupled to a complementing entity. In a preferred aspect this refers to addition of FE-AA, standard amino acid, non-standard amino acid, pseudo-amino
20    acids or precursors thereof to a tRNA or pre-tRNA or tRNA like structure by a chemical or enzymatic reaction.

Hence, the term "charged tRNA" refers to a tRNA covalently attached to a spacer, such as a FE-AA, standard amino acid, non-standard amino acid, pseudo-amino
25    acids or precursors thereof.

<u>Cleavable linker</u>

A cleavable linker according to the present invention is a residue or chemical bond
30    capable of being cleaved under specific predetermined conditions. Preferably, cleavable linkers are separating a spacer and a functional entity of a first building block.

Non-limiting examples of cleavable linkers that may be employed with the present invention are given in figure 10.

5      Coding elements

Coding elements according to the present invention comprises at least one recognition group capable of recognising a predetermined complementing element. Preferably, one particular coding element is capable of specifically interacting with
10     the predetermined complementing element, and accordingly the coding element preferably is not capable of interacting with other complementing elements, or interacts with less efficiency with these other complementing elements.

Coding elements may be selected from the group consisting of first coding elements
15     and second coding elements.

First coding element comprises at least one recognition group capable of recognising a predetermined first complementing element.

20     Second coding element comprises at least one recognition group capable of recognising a predetermined second complementing element.

In one preferred embodiment of the present invention the coding elements are selected from the group consisting of nucleotides, nucleotide derivatives, nucleotide
25     analogues, and any combination thereof, such as oligonucleotides or oligonucleotide analogues. Preferably, nucleotides are ribonucleic acids comprising a base selected from adenine (A), uracil (U), guanine (G), and cytosine (C) and derivatives and analogues thereof.

30     Each coding element may consists of any desirable number of nucleotides, nucleotide derivatives and/or nucleotide analogues, for example 1, such as 2, for example 3, such as 4, for example 5, such as 5 to 10, for example 10 to 15, such as 15 to 20, for example more than 20 nucleotides, nucleotide derivatives and/or nucleotide analogues. Preferably, each coding element consists of 3 of nucleotides,

nucleotide derivatives and/or nucleotide analogues. More preferably, the coding element is a codon.

Coding elements according to the present invention must be capable of serving as a template for a ribosome mediated incorporation of subunits into a polypeptide, a polypeptide derivative or a polypeptide analogue.

Codon

A codon is a sequence of 3 ribonucleotides that encodes a particular amino acid in a messenger RNA molecule.

Complementing entity

A complementing entity according to the present invention is an entity that comprises one complementing element. Preferably a complementing entity according to the present invention comprises only one complementing element.

In one preferred embodiment of the present invention the complementing element comprises and even more preferably consists of nucleotides and/or nucleotide analogues. For example nucleotides and nucleotide analogues selected from the group consisting of DNA, RNA, LNA, PNA and mixtures thereof.

In one preferred embodiment of the present invention the complementing entity is a tRNA or tRNA-like structure. By tRNA like structure is meant any structure, which can perform the function of tRNA, that is transfer of a standard amino acid, non-standard amino acid or pseudo amino acid to a template for ribosome mediated synthesis of a polypeptide, a polypeptide derivative or a polypeptide analogue.

In one embodiment of the present invention the complementing entity is a tRNA. In another embodiment of present invention the complementing entity is a pseudoknot.

## Complementing element

Complementing elements according to the present invention comprise at least one recognition group capable of recognising a predetermined coding element. Preferably, one particular complementing element is capable of specifically interacting with the predetermined coding element, and accordingly the complementing element preferably is not capable of interacting with other coding elements, or interacts with less efficiency with these other coding elements.

A complementing element according to the present invention may be selected from the group consisting of first complementing elements and second complementing elements

First complementing elements according to the present invention comprise at least one recognition group capable of recognising a predetermined first coding element. When comprised within a building block, a first complementing element is comprised within a first building block.

Second complementing elements according to the present invention comprise at least one recognition group capable of recognising a predetermined second coding element. When comprised within a building block, a second complementing element is comprised within a second building block.

In one preferred embodiment of the present invention the complementing elements are selected from the group consisting of nucleotides, nucleotide derivatives, nucleotide analogues, and any combination thereof, such as oligonucleotides or oligonucleotide analogues.

Each complementing element may consist of any desirable number of nucleotides, nucleotide derivatives and/or nucleotide analogues. For example each complementing element may consist of 1, such as 2, for example 3, such as 4, for example 5, such as 5 to 10, for example 10 to 15, such as 15 to 20, for example more than 20 nucleotides, nucleotide derivatives and/or nucleotide analogues.

Preferably, the nucleotides may be selected from the group consisting of ribonucleic acids comprising a base selected from adenine (A), uracil (U), guanine (G), and cytosine (C) and derivatives and analogues thereof.

5      In one embodiment the complementing element may be an anticodon.

Decoding

10     The process during translation in which the complementing element of a building block interacts, by hybridisation, with the coding element of a template, thereby facilitating synthesis of a spacer backbone mediated by the catalytic activity of a ribosome.

15     For example decoding may be the process during translation wherein an anticodon of a tRNA molecule hybridise to the complementary codon of a template sequence thereby facilitating the ribosome mediated formation of a covalent bond between two spacer reactive groups.

Functional entity (FE)

20

A functional entity according to the present invention comprises a functional group(s) and functional entity reactive group(s) capable of linking adjacently positioned functional groups. Functional entities are in general forming part of a first building block.

25

The functional entity may comprise any desirable number of functional entity reactive groups, for example the functional entity may comprise more at leastone, such as 2, for example 3, such as 4, for example 5, such as more than 5 functional entity reactive groups.

30     Within a first building block the functional entity may be separated from the spacer by a cleavable linker or by a selectively cleavable linker. The cleavable linker or the selectively cleavable linker may be attached covalently to the functional entity, for example the cleavable linker or the selectively cleavable linker may be attached

covalently to a functional entity reactive group or to a functional group of said functional entity.

Non-limiting examples of functional entities are given in figure 25.

FE-AA

FE-AA designates a functional entity amino acid, i.e. an amino acid covalently linked to a functional entity.

Functional entity reactive groups

Functional entity reactive groups are reactive groups comprised within a functional entity.

Corresponding functional entity reactive groups are a pair of reactive groups of different functional entities, which are capable of forming a chemical bond linking one functional group with another functional group either directly or through a linker. Non-limiting examples of pairs of functional entity reactive groups and chemical bonds that may be formed by reaction of said groups are given in figure 29.

The functional entity reactive groups may for example be selected from the group consisting of N-carboxyanhydride (NCA), N-thiocarboxyanhydride (NTA), amine, carboxylic acid, ketone, aldehyde, hydroxyl, thiol, ester, thioester, any conjugated system of double bonds, hydrazine, N-hydroxysuccinimide ester, and epoxide.

Furthermore, the functional entity reactive group may be an electrophile or the functional entity reactive group may be a nucleophile or the functional entity reactive group may be a radical.

## Functional group

5      A functional group is a group forming part of a templated molecule. The sequence identity of functional groups in a templated molecule is a result of the capability of the template to template the synthesis of the templated molecule.

Non-limiting examples of functional groups are given in figure 24.

10     ## Neighbouring

Elements, groups, entities or residues consecutive to one another in a sequence are said to be neighbouring. In particular, spacers are neighbouring, when they are part of building blocks, which comprise complementing elements, which recognise

15     coding-elements that are located in sequence on a template, when said coding elements are complemented with said complementing elements.

Accordingly, preferably every spacer may have a maximum of two neighbouring spacers.

20

## Non-amino acid.

Chemical entity not capable of being incorporated into a peptide by ribosome mediated translation

25     ## Non-standard amino acid

A non-standard amino acid is capable of being incorporated into a peptide or peptide like structure by translation mediated by a ribosome.

30     A non-standard amino acid according to the present invention is any amino acid comprising an amino group and a carboxyl group separated by an α-carbon. The amino acid may for example be selected from the group consisting of, Aib, Nal, Sar,

Orn, Lysine analogues DAP and DAPA or any of the amino acids described in US 5,573,905. Furthermore, non-standard amino acids may be any of the above mentioned or any standard amino acids which further comprises one or more moieties selected from the group consisting of hydroxyl, bromo, fluoro, chloro, iodo, mercapto, thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, nitro, amino, alkoxyl and/or amido.

The non-standard amino acid is capable of being incorporated into a peptide or peptide like structure by translation mediated by a wt, mutant, modified or recombinant ribosome.

## Nucleotides

Nucleotides according to the invention includes ribonucleotides comprising a nucleobase selected from the group consisting of adenine (A), uracil (U), guanine (G), and cytosine (C), and deoxyribonucleotide comprising a nucleobase selected from the group consisting of adenine (A), thymine (T), guanine (G), and cytosine (C).

Nucleobases are capable of associating specifically with one or more other nucleobases via hydrogen bonds. Thus it is an important feature of a nucleobase that it can only form stable hydrogen bonds with one or a few other nucleobases, but that it can not form stable hydrogen bonds with most other nucleobases usually including itself. The specific interaction of one nucleobase with another nucleobase is generally termed "base-pairing".

The base pairing results in a specific hybridisation between predetermined and complementary nucleotides. Complementary nucleotides according to the present invention are nucleotides that comprise nucleobases that are capable of base-pairing.

Of the naturally occurring nucleobases adenine (A) pairs with thymine (T) or uracil (U); and guanine (G) pairs with cytosine (C). Accordingly, e.g. a nucleotide comprising A is complementary to a nucleotide comprising either T or U, and a nucleotide comprising G is complementary to a nucleotide comprising C.

## Nucleotide analogues

Nucleotide analogues include any nucleotide analogues capable of specific bas-
pairing, for example derivatives of naturally occurring nucleotides or nucleotide
analogues wherein the nucleotide backbone differs from naturally occurring
nucleotide backbones.

## Pseudo-amino acid

An entity comprising a substituted amino group or/and carboxyl group separated by
an $\alpha$-carbon or $\alpha$-amine capable of being incorporated into a peptide by ribosomes.

For example, a pseudo amino acid may comprise a thiol group and a carboxyl group
separated by an $\alpha$-carbon resulting in a thioester bond in the backbone.

Examples of pseudo-amino acids are given in figure 3.

## Reactive group

Reactive groups are groups that are capable of reacting chemically with a
corresponding reactive group after being brought into reactive contact herewith.

For example reactive groups according to the present invention may be selected
from the group consisting of di-coumarin, carboxylic acids, di-carboxylic acid,
hydroxyls, diols, thioesters, amines, primary amines, secondary amines, di-amine,
halogens, isocyanate, $\alpha$-haloacetyl, UDP-glucose, UDP-activated saccharides,
glucosyl sulphide/sulfoxide activation system (Kahne glucosylation), N-
hydroxysuccinimide ester, carboxyanhydride (5-membered ring), carboxyanhydride
(6 membered ring), carboxyanhydride (7-membered ring), 2,2-diphenylthiazinanone
(5-membered ring), 2,2-diphenylthiazinanone (6-membered ring), 2,2-
diphenylthiazinanone (7-membered ring), sulfonic acid, diaminophosphine, epoxide,
thioepoxide, thiol, aldehyde, hydroxylamine, alkyl sulfonate, alkene, di-diene,

vinylchloride unit, styrene-unit and ethylene unit. However, any other suitable reactive group, which does not impair or destroy the template may also be employed with the present invention. Examples of pairs of reactive groups are given in figure 29.

5    Recognition group

A recognition group may be part of a coding element or a complementing element. Recognition groups are involved in the recognition of a complementing element capable of recognising a coding element or in the recognition of a coding element

10    capable of recognising a complementing element. Preferred recognition groups are natural and non-natural nitrogeneous bases of a naturally occurring or non-natural nucleotide.

Ribosome

15

A ribosome according to the present invention is any ribosome capable of catalysing a reaction forming a covalent coupling of two amino acids selected from the group consisting of standard amino acids, non-standard amino acids, pseudo-amino acids and precursors thereof.

20

Preferably, the reaction is templated by an mRNA, to which two tRNAs, each carrying one of said standard amino acids, non-standard amino acids, pseudo-amino acids or precursors thereof . In one embodiment the covalent coupling is formation of an amide bond or an ester- or thioester bond. However, the bond could

25    be any bond formed by the reaction between a carbonyl and a nucleophile. The catalysis is dependent on the interaction between template coding elements and the complementing elements of building blocks.

In one embodiment of the invention, ribosomes are capable of catalysing the

30    reaction between amino acid residues, whereby a peptide is formed.

The ribosome responsible for the incorporation of spacers according to the present invention may be any useful ribosome known to the person skilled in the art. For example the ribosome may be a wild type ribosome or a mutant, recombinant or

otherwise modified ribosome obtained from or produced by one or more suitable organism(s). One or more components of the ribosome may be synthesised in vitro by any suitable procedure such as solid-phase protein or nucleic acid chemistry.


5      Scaffold


A scaffold is a moiety comprising at least 3 reactive groups capable of reacting with functional entity reactive groups, thereby forming covalent bonds between said scaffold and said functional entities.

10

In one embodiment the scaffold is a functional entity incorporated into the spacer backbone by ribosome mediated translation. In a second embodiment of this invention the scaffold is attached covalently or non-covalently to any part of the template complexes that is not the spacer backbone. In some cases it may be

15     beneficial to have the scaffold located externally such as on a solid support, Or the scaffold may be located in solution Furthermore, the scaffold may comprise one or more cleavable linkers, and /or the scaffold may be attached to the template or complementing template through a cleavable linker.


20     Selectively cleavable linker


Selectively cleavable linkers are not cleavable under conditions wherein a cleavable linker is cleaved. Accordingly, it is possible to cleave the cleavable linkers separating complementing entities and functional entities in a templated molecule

25     without at the same time cleaving selectively cleavable linkers separating - in the same templated molecule - a subset of complementing entities and functional entities. It is thus possible to obtain a complex comprising a templated molecule and the template that has directed the templated- synthesis of the templated molecule, wherein the template and the templated molecule are linked by one or more,

30     preferably one, selectively cleavable linker(s).


Non-limiting examples of cleavable linkers are given in figure 10. Selectively cleavable linkers should be selected according to the cleavable linkers of the specific embodiment of the invention. For example if the cleavable linkers are

cleavable by alkali treatment, the selectively cleavable linkers may for example be selected from the group consisting of linkers cleavable by photocleavage, acid cleavage, catalytic cleavage enzymatic cleavage and temperature cleavage.

5      Spacer

A spacer is a group forming part of a building block. A spacer according to the present invention comprises at least one spacer reactive group.

10     Spacers which are part of a first building block are separating the functional entity and the complementing entity of said first building block. The spacer may be covalently attached to the complementing entity of said first building block. The spacer may also be covalently attached to the functional entity of said building block, however, preferably the spacer is attached to the functional entity via a cleavable or
15     selectively cleavable linker.

Spacers which are part of second building blocks may be covalently attached to the complementing entity of said second building block.

20     In one embodiment of the present invention the spacer may be selected from the group consisting of amino acids, for example the spacer may be an α-amino acid or the spacer may be selected from the group consisting of standard amino acids, non-standard amino acids, pseudo-amino acids and derivatives thereof.

25     In one embodiment of the inventionthe spacer consists of a standard amino acid residue including the entire side-chain and preferably, the spacer does not form part of the templated molecule. Accordingly, said standard amino acid residue may form part of the spacer backbone, but preferably does not form part of the templated molecule except for the cases in which the standard amino acid comprise a
30     selectively cleavable linker of the templated molecule connecting the templated molecule and a spacer backbone unit.

Each spacer comprises at least one spacer reactive group, however a spacer may also comprise at least 2, such as 2, for example 3, such as more than 3 spacer reactive groups. Preferably, each spacer comprises at least 2 spacer reactive groups.

5

In one preferred embodiment one or more spacer reactive groups may be selected from the group consisting of acyls and amines. Preferably at least one spacer reactive group is selected from the group consisting of acyl and at least one spacer reactive reactive group is selected from the group consisting of amines. Accordingly,

10    it is preferred that each spacer comprises one spacer reactive group, which is an · acyl and another spacer reactive group which is an amine.


Spacer backbone


15    A spacer backbone according to the present invention is preferably formed by linking, by means of a reaction involving spacer reactive groups, neighbouring building block spacers. Hence, a preferred spacer backbone according to the present invention comprises, or more preferably consists of at least 3 spacer residues.

20    Neighbouring building block spacers are linked by a ribosome mediated reaction involving spacer reactive groups. Preferably, the reaction involves a direct chemical reaction between spacer reactive groups of neighbouring building blocks that results in the formation of a chemical bond between said two neighbouring spacers.

25    The chemical bond that links two neighbouring spacers may be of any suitable kind, for example the bond may be selected from the group consisting of amide bonds, aryl, acyl, ester and, thioester bonds. Examples of reactive groups are given in figure 3.

30    In one preferred embodiment of the present invention linking neighbouring building block spacers consists of the formation of an amide-bond. In particular, this is relevant in embodiments wherein at least one spacer reactive group of one spacer is · an acyl and at least one spacer reactive group of the neighbouring spacer is an amine.

The spacer residues comprised within a spacer backbone according to the invention may be directly attached to a functional entity. Preferably, a spacer backbone according to the present invention comprises at least 3 spacers that are directly attached to a functional entity.

In one embodiment of the present invention the spacer backbone only comprises spacer residues that are directly attached to a functional entity.

In another embodiment of the present invention the spacer backbone comprises spacer residues that are directly attached to a functional entity, wherein every two spacer residues that are directly attached to a functional entity are separated by a minimum of 0 spacer residues that are not directly attached to a functional entity, for example at least 1, such as at least 2 first, for example around 2, such as around 3, for example around 4, such as around 5, for example around 6, for example around 7, such as around 8 to 10, for example around 10 to 15, such as around 15 to 20, for example around 20 to 30 spacer residues, that are not directly attached to a functional entity.

In general, the spacer backbone may be a linear sequence of spacers.

In some embodiments of the present invention it is desirable that the spacer backbone adopts a predetermined and relatively stable 3 dimensional shape after formation. This could happen gradually during synthesis of the spacer backbone or it could happen after synthesis of the entire spacer backbone. The purpose of a predetermined 3 dimensional shape of the spacer backbone may for example be to bring two predetermined functional entities close together, so that they become adjacently positioned and a functional entity reactive group of one of said two functional entities may react with a functional entity reactive group of the other of said two functional entities.

The spacer backbone may adopt any useful predetermined 3 dimensional shape. In one example the spacer backbone may for instance have the form of an α-helix. Alternatively the spacer backbone may have the form of a coiled coil. Furthermore, the spacer backbone may for example have a form selected from the group

consisting of β-sheets, beta-turn, beta-helix, coiled coils, helix-turn helix, part of a collagen structure and zinc finger structures. In addition the spacer backbone may have a 3 dimensional shape that comprises different kinds of structures, for example one or more selected from the group consisting of an α-helix, a β-sheet, coiled coil,

5 · beta-turn, beta-helix, helix-turn helix, part of a collagen structure and zinc finger structures.

In one embodiment the spacer backbone is denatured and bound to a solid surface that determines the shape of the spacer backbone. For example the spacer

10 backbone may be a denatured polypeptide or derivative thereof or a denatured polypeptide like structure. The solid surface may for example be a glass surface, a plastic surface or a mineral surface,

In one embodiment when the spacer backbone has the form of an α-helix and/or

15 comprises part(s) which have the form of an α-helix, the spacer backbone preferably comprises one functional entity per helical turn of the spacer backbone. For example the spacer backbone may comprise a spacer directly attached to a functional entity for every 4 spacer residue, such as every 7 spacer residue, such as every 11 residue etc.

20

In one embodiment of the present invention the spacer backbone may be formed by a ribosome mediated incorporation of spacers. In particular, in embodiments of the present invention, wherein spacers are selected from the group consisting of amino acids, the spacer backbone may be formed by a ribosome mediated incorporation of

25 amino acids.

The ribosome, that perform the incorporation of spacers may be any useful ribosome known to the person skilled in the art. For example the ribosome may be a wild type, mutant, modified or recombinant ribosome derived from any organism but

30 preferably from *Escherichia coli.*

Spacer reactive groups

Spacer reactive groups according to the present invention are reactive groups (see
5    herein above) comprised within a spacer. In particular, corresponding spacer
reactive groups when brought into reactive contact with each other are capable of
forming a chemical bond linking one spacer to a neighbouring spacer.

In one preferred embodiment of the present invention the spacer reactive groups are
10    selected from the group consisting of acyls and amines.

Standard amino acids/residues

Throughout the description and claims either the three letter code or the one letter
15    code for standard amino acids are used. Where the L or D form has not been
specified it is to be understood that the amino acid in question may have the natural
L form, cf. Pure & Appl. Chem. Vol. (56(5) pp 595-624 (1984) or the D form.

In particular standard amino acids may be selected from the group consisting of Ala,
20    Val, Leu, Ile, Pro, Phe, Trp, Met, Gly, Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys,
Arg and His.

Standard amino acid residues include residues of any of the above mentioned
amino acids, that is any of the above mentioned amino acids, which have been
25    reacted with at least one other species, such as 2, for example 3, such as more than
3 other species.

Template

30    A template according to the present invention preferably comprises a sequence of n
coding elements selected from the group consisting of first coding elements and
second coding elements, wherein n is an integer of more than 2. More preferably,
the template comprises at least 3 first coding elements.

n is preferably an integer of more than 1 and less than 1000, for example between 5 and 500, such as between 10 and 100, for example between 3 and 100, such as between 3 and 50.

In one embodiment the template comprises a ratio of first coding elements to second coding elements of 50:1, such as 40:1, for example 30:1, such as 25:1, for example 20:1, such as 15:1, for example 10:1, such as 8:1, for example 6:1, such as 5:1, for example 4:1, such as 3:1, for example 2:1, such as 1:1, for example 1:2, such as 1:3, for example 1:4, such as 1:5, for example 1:6, for example 1:7, such as 1:8, for example 1:10, such as 1:15, for example 1:20, such as 1:25, for example 1:30, such as 1:40, for example 1:50. However, the template may also only comprise first coding elements and no second coding elements.

The template preferably comprises at least 1, for example at least 2, such as at least 3, for example at least 4, such as at least 5, for example at least 10, for example at least 15, such as at least 20, for example at least 30, such as at least 40, for example at least 50, such as at least 75, for example at least 100, such as at least 150, for example at least 200 elements first coding elements.

Furthermore the template may comprise at least 1 for example at least 2, such as at least 3, for example at least 4, such as at least 5, for example at least 10, for example at least 15, such as at least 20, for example at least 30, such as at least 40, for example at least 50, such as at least 75, for example at least 100, such as at least 150, for example at least 200 second coding elements.

The first and the second coding elements may be dispersed between each other on the template in any desirable order.

In one preferred embodiment of the present invention, the template may be nucleic acid or nucleic acid analogue. Preferably, the template may be a nucleic acid, which can be template of a ribosome mediated translation.

More preferably, the template comprises or consists of RNA or a derivative or analogue thereof. For example, the template may comprise RNA residues that are

modified on the 2' position of the ribose moiety. In some embodiments of the invention it may be preferred that the template is capped RNA. Most preferably, the template is mRNA.

5      In one embodiment of the present invention the template preferably comprises at least one stop coding element. For example, no corresponding complementing element may be present that can hybridise to said stop coding element.

## Templated synthesis

10

Templated synthesis is the process, wherein a templated molecule comprising at least 3 covalently linked functional entities is synthesised. The identity of each of the functional entities that the templated molecule comprises is defined by a predetermined template sequence.

15

Prior to the formation of the templated molecule, the functional entity is incorporated by ribosome mediated translation of a predetermined template sequence. Formation of the spacer backbone requires decoding of the template sequence by specifically charged tRNAs and the peptidyl transferase function of the ribosome. Consequently,

20     the translation step involving template decoding and the peptidyl transferase activity of the ribosome does *not* produce the templated molecule per se but is required for choosing the functional entities that are to be assembled into the templated molecule. The covalent coupling of functional entities may occur concommitantly with the translation step or after the partial or complete synthesis of the spacer

25     backbone in a reaction step(s) that is separate from the peptidyl transferase reaction step of the ribosome. Finally one or more of the linkages connecting one or more functional entities with the spacer backbone may be cleaved.

Accordingly, the templated incorporation of functional entities and their coupling to
30     each other can take place without change in conditions, or addition of further reagents or catalysts, to the system, i.e. it can take placein a closed system. Consequently the process of choosing the functional entities according to the template sequence does not require further intervention once the process has been initiated.

However, the cleavage of the linker that links the functional entities and the spacer backbone may in some embodiments require the addition of further reagents, for example cleaving reagents.

5 In particular, templated synthesis involves contacting a sequence of coding elements with particular complementing elements. Accordingly, there is a predetermined one to one relationship between the identity of functional groups of the templated molecule and the sequence identity of first coding elements of the template that templated the synthesis of the templated molecule. Thus, during the

10 templated synthesis of the templated molecule, a functional group is initially contacting - by means of a spacer and/or a complementing element, or otherwise - the coding element capable of templating that particular functional group into the templated molecule. An oligonucleotide templated synthesis is based on an interaction of each nucleotide with its pairing partner in the template in a one-base-

15 to-one-base pairing manner. The interaction specifies the incorporation of complementing nucleotides opposite their base pairing partners in the template. Consequently, one base, including a heterocyclic base, from each oligonucleotide strand interact when forming specific base-pairs. This base pairing specificity may be achieved through Watson-Crick hydrogen-bonding interactions between the

20 bases, where the bases may be natural (i.e. A, T, G, C, U), and/or non-natural bases such as those e.g. disclosed e.g. in US 6,037,120, incorporated herein by reference. Further examples of non-natural bases nucleotides are e.g. PNA (peptide nucleic acid), LNA (locked nucleic acid) and morpholinos. Base pairing of oligonucleotides containing non-standard base pairs can be achieved by other

25 means than hydrogen bonding (e.g. interaction between hydrophobic nucleobases with "complementary" structures; Berger et al., 2000, Nucleic Acids Research, 28, pp. 2911-2914). The interacting oligonucleotide strands as well as the individual nucleotides are said to be complementary. The specificity of the interaction between oligomers results from the specific base pairing of a nucleotide with

30 another nucleotide or a predetermined subset of nucleotides, for example A base pairing with U, and C base pairing with G.

Templated molecule

A templated molecule within the scope of the present invention is a molecule comprising a plurality of covalently linked functional groups, wherein the templated molecule is obtainable by templated synthesis using the template.

In one embodiment of the present invention the templated molecule preferably comprises or essentially consists of amino acids selected from the group consisting of α-amino acids, β-amino acids, γ-amino acids, ω-amino acids.

For example, the templated molecule may comprise or essentially consist of α-amino acids, such as for example non-substituted, monosubstituted or disubstituted α amino acids.

In another example the templated molecule may comprise or essentially consist of monosubstituted β-amino acids, disubstituted β-amino acids, trisubstituted β-amino acids and/or tetrasubstituted β-amino acids. In one embodiment the backbone structure of said β-amino acids may comprise or essentially consist of a cyclohexane-backbone and/or a cyclopentane–backbone.

In yet another example the templated molecule may comprise or essentially consist of γ-amino acids.

In a still further example the templated molecule may comprise or essentially consist of ω-amino acids.

Furthermore, the templated molecule may for example comprise or essentially consist of vinylogous amino acids or the templated molecule may for example comprise or essentially consist of N-substituted glycines.

Accordingly, the templated molecule according to the present invention may comprise any of a variety of different subunits. For example the templated molecule may comprise or essentially consist of functional groups and/or functional entities selected from the group of α-peptides, β-peptides, γ-peptides, ω-peptides, mono-, di- and tri-substituted α-peptides, β-peptides, γ-peptides, ω-peptides, peptides wherein the amino acid residues are in the L-form or in the D-form, vinylogous polypeptides, glycopoly-peptides, polyamides, vinylogous sulfonamide peptide, polysulfonamide, conjugated peptides comprising e.g. prosthetic groups, polyesters, polysaccharides, polycarbamates, polycarbonates, polyureas, polypeptidylphosphonates, polyurethanes, azatides, oligo N-substituted glycines, polyethers, ethoxyformacetal oligomers, poly-thioethers, polyethylene glycols (PEG), polyethylenes, polydisulfides, polyarylene sulfides, polynucleotides, PNAs, LNAs, morpholinos, oligo pyrrolinone, polyoximes, polyimines, polyethyleneimines, polyimides, polyacetals, polyacetates, polystyrenes, polyvinyl, lipids, phospholipids, glycolipids, polycyclic compounds comprising e.g. aliphatic or aromatic cycles, including polyheterocyclic compounds, proteoglycans, and polysiloxanes, including any combination thereof.

Adjacently positioned residues within a templated molecule according to the present invention may be linked by any useful chemical bond, for example adjacently positioned residues of the templated molecule may be linked by a chemical bond selected from the group of chemical bonds consisting of peptide bonds, sulfonamide bonds, ester bonds, saccharide bonds, carbamate bonds, carbonate bonds, urea bonds, phosphonate bonds, urethane bonds, azatide bonds, peptoid bonds, ether bonds, ethoxy bonds, thioether bonds, single carbon bonds, double carbon bonds, triple carbon bonds, disulfide bonds, sulfide bonds, phosphodiester bonds, oxime bonds, imine bonds, imide bonds, including any combination thereof.

The backbone structure of the templated molecule will depend on the nature of the functional groups comprised within said templated molecule as well as the chemical bonds connecting functional groups of a templated molecule. Accordingly, the backbone structure of said templated molecule may for example comprise or essentially consist of a molecular group selected from -NHN(R)CO- ; -NHB(R)CO- ; -NHC(RR')CO- ; -NHC(=CHR)CO- ; -NHC$_6$H$_4$CO-; -NHCH$_2$CHRCO-; -NHCHRCH$_2$

CO- ; -COCH$_2$- ; -COS- ; -CONR- ; -COO- ; -CSNH- ; -CH$_2$ NH- ; -CH$_2$CH$_2$- ; -CH$_2$ S-
; -CH$_2$ SO- ; -CH$_2$SO$_2$- ; -CH(CH$_3$)S- ; -CH=CH- ; -NHCO- ; -NHCONH- ; -CONHO- ;
-C( =CH$_2$)CH$_2$- ; -PO$_2^-$NH- ; -PO$_2^-$CH$_2$- ; -PO$_2^-$CH$_2$N$^+$- ; -SO$_2$NH$^-$ ; and lactams.

A templated molecule according to the present invention may comprise any desirable number of functional groups. The functional groups of a templated molecule may all be identical, however it is also contained within the present invention that the templated molecule comprises different functional groups. For example, the templated molecule according to the invention may comprise or essentially consist of at least 2 different functional groups, such as at least 3 different functional groups, for example at least 4 different functional groups, such as at least 5 different functional groups, for example at least 6 different functional groups, such as at least 7 different functional groups, for example at least 8 different functional groups, such as at least 9 different functional groups, for example at least 10 different functional groups, such as more than 10 different functional groups.

## Template/templated molecule complex

A template/templated molecule complex is a complex comprising two parts, wherein one part templates the synthesis of the other part. Hence, one part may be synthesised by templated synthesis of the other part.

## tRNA and tRNA like structures

The term tRNA according to the present invention includes any naturally occurring transfer RNA. tRNA like structures includes any molecule capable of performing the function of a tRNA, i.e. bringing a standard and/or non-standard and/or pseudo amino acid into contact with a template and thereby enabling ribosome mediated incorporation of said amino acid, non-standard- or pseudo-amino acid.

## Detailed description of the invention

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### Synthesis of templated molecules

Herein above are a number of prior art procedures for synthesis and functional selection of peptides, for example peptide display and PROfusion. However, in all
10 practical terms, the described procedures are all limited to the synthesis and functional selection of $\alpha$-peptides.

The present invention is not restricted by the above-mentioned limitations and may be employed for the synthesis of templated molecules of diverse backbone and
15 residue chemistry.

The present invention describes methods for synthesizing templated molecules and/or complexes and methods for targeting such molecules and/or complexes to a target species. Furthermore, the invention describes methods for amplification of
20 templated molecules by amplification of templates that specifies selected templated molecules.

The templated molecules according to the present invention may be specified by a template comprising n coding elements, selected from the group consisting of first
25 coding elements and second coding elements, wherein n is an integer of more than 1, such as 2, for example 3, such as 4, for example 5, such as 6, for example7, such as 8, for example 9, such as 10, for example in the range from 10 to 15, such as in the range from 15 to 20, for example in the range from 20 to 30, such as in the range from 30 to 40, for example in the range from 40 to 50, such as in the range
30 from 50 to 100, for example in the range from 100 to 250, such as in the range from 250 to 500, for example n may be an integer larger than 500. In the present invention the template that encode said templated molecule is physically attached either covalently or non-covalently to the templated molecules by a technique(s)

known to those persons skilled in the art (Peptide display, PROFusion or other means).

Hence, in one embodiment of the invention the template is tethered to a molecular
5    entity capable of forming a covalent bond to the templated molecule. For example said molecular entity may be puromycin.

The templated molecules are preferably synthesised from second building blocks comprising a functional entity comprising a functional group and one or more
10    functional entity reactive group(s) capable of reacting chemically with other functional entity reactive group(s) to form covalent bonds, linking functional groups of adjacent functional entities and thereby forming a templated molecule. The functional entity of a second building block may be separated from a complementing element of said building block and a spacer of said building block by a cleavable
15    linker, or a selectively cleavable linker. The complementing element is preferably capable of complementing a predetermined coding element of the template and is preferably specific for said coding element.

Following complementation of a coding element by a complementing element, each
20    complementing element will present an appended group capable of being linked by spacer reactive group(s) to a neighbouring spacer presented by a neighbouring complementing element. Consecutive reactions of appended spacer of complementing elements involving spacer reactive groups of neighbouring complementing elements produce a spacer backbone exposing functional entities
25    separated from said spacer backbone by said cleavable or selectively cleavable linker(s).

Subsequent to spacer backbone synthesis adjacently positioned reactive groups of adjacently positioned functional entities are reacted to form a polymer or a branched
30    molecule connected to the spacer backbone by functional groups and said cleavable or selectively cleavable linkers.

Cleavable linkers are cleavable under conditions wherein a selectively cleavable linker is not cleavable. Accordingly, it is possible to cleave the cleavable linkers
35    linking the spacer backbone and functional groups in a templated molecule without

at the same time cleaving selectively cleavable linkers. It is thus possible to obtain a complex comprising a templated molecule and the spacer backbone still attached to the template that directed the synthesis of the templated molecule wherein the templated molecule and the spacer backbone are linked by one or more, preferably one, selectively cleavable linker(s).

In a further aspect it may be desirable to cleave;

- none of the cleavable linkers or,
- to cleave only a subset of cleavable linkers.

As each complementing element is capable of recognising a predetermined coding element of a template, and as each coding element in turn defines a predetermined functional group, the sequence of coding elements of the template will template the synthesis of the templated molecule comprising a predetermined plurality of covalently linked functional groups.

Following formation of the templated molecules, the molecules of desired properties are isolated by a selection procedure screening for relevant molecular characteristics.

The generation of additional templated molecules can be directed from the template appended each templated molecule without any need for sequencing or any other form of characterisation. Accordingly, the complexes of the invention comprising a templated molecule linked to the template that specifies said molecule enable rapid selection and amplification of templated molecules with relevant properties.

In a first aspect, the present invention provides a method for synthesising a templated molecule comprising a plurality of functional groups, said method comprising the steps of

i)      providing at least one template comprising a sequence of n coding elements selected from the group consisting of first coding elements and second coding elements,

wherein each coding element comprises at least one recognition group capable of recognising a predetermined complementing element, and

wherein n is an integer of at least 3,

with the proviso that the template comprises at least 3 first coding elements,

ii)    providing a plurality of building blocks selected from the group consisting of first building blocks and second building blocks, with the proviso that at least 3 first building blocks are provided,

wherein each first building block comprises

a)  at least one complementing entity comprising a first complementing element comprising at least one recognition group capable of recognising a predetermined first coding element,

b)  at least one functional entity comprising at least one functional group and at least one functional entity reactive group, and

c)  at least one spacer comprising at least one spacer reactive group, wherein the spacer is separating the at least one functional entity from the at least one complementing entity, and

wherein each second building block comprises

a)  at least one complementing entity comprising a second complementing element comprising at least one recognition group capable of recognising a predetermined second coding element,

b)  and at least one spacer comprising at least one spacer reactive group,

iii)    complementing coding elements by contacting each coding element with a building block complementing element capable of recognising said coding element, wherein at least 2 coding elements are interacting with complementing elements simultaneously,

with the proviso that a total of at least 3 first coding elements are complemented; and

iv)    forming a spacer backbone by linking neighbouring spacers in a ribosome catalysed reaction by means of reacting spacer reactive groups, and

v)    obtaining a templated molecule comprising at least 3 covalently linked, functional groups by linking, by means of reactioning functional entity reactive groups, a functional group of one functional entity to a functional group of another adjacently positioned, functional entity and linking said other functional entity to yet another adjacently positioned functional entity.

In a preferred aspect of the invention the templated molecule comprises a sequence of at least 3 covalently linked functional groups.

It is comprised within the present invention that the method comprises more step(s) in addition to the above mentioned steps. In addition each step may comprise a number of sub-steps, not explicitly mentioned herein above.

For example, in one embodiment of the present invention step iii) to iv) of the above mentioned method comprises the steps of

a)    complementing 2 neighbouring coding elements simultaneously by contacting each coding element with a building block complementing element capable of recognising said coding element, and

b)    forming a spacer backbone by linking, by means of a reaction involving spacer reactive groups, the 2 building block spacers, and

c) complementing at least one further neighbouring predetermined coding element by contacting said coding element with a building block complementing element capable of recognising said coding element, and

d) elongating the spacer backbone by linking to the spacer backbone, by means of a reaction involving spacer reactive groups, the neighbouring building block spacer.

The steps of the above mentioned methods may be performed in any given timely order. For example, all coding elements may be complemented before reacting neighbouring spacer reactive groups to form a spacer backbone. Alternatively, only two, such as 3, for example 4, such as 5 coding elements may be complemented before reacting neighbouring spacer reactive groups to form a spacer backbone.

It is furthermore comprised within the present invention that the templated molecule may be formed by linking adjacent functional groups, subsequent to formation of the entire spacer backbone. Alternatively, however as soon as the spacer backbone comprises spacers of 2, such as 3, for example 4, such as 5 first building blocks, then the functional entities of said 2, such as 3, for example 4, such as 5 first building blocks may be linked to each other by reactions involving adjacent functional entity reactive groups to form a templated molecule. Subsequently, the spacer backbone may be elongated by addition of further first building block spacers and the templated molecule may accordingly be elongated, by adding functional entities of said first building blocks to the templated molecule.

In one preferred embodiment the steps of the methods are performed chronologically in the mentioned order.

The individual steps of the methods may be performed any number of desirable times. For example, steps c) and d) may be repeated at least twice, such as repeated at least three times, for example at least 4 times, such as at least 5 times, for example at least 10 times, for example at least 15 times, such as at least 20 times, for example at least 30 times, such as at least 40, for example at least 50, such as at least 75 times, for example at least 100 times, such as at least 150 times,

for example at least 200 times. Alternatively, steps c) and d) may be repeated between 2 and preferably 10,000 times, for example between 5 and preferably 1000 times, such as between 10 and preferably 500 times.

In addition to the above mentioned steps, it is frequently desirable to break one or more chemical bonds, for example to liberate the templated molecule from the spacer backbone or to liberate the spacer backbone from the template or to liberate the spacer backbone from individual complementing entities.

In one embodiment the method furthermore comprises the step of

vi)     breaking the covalent bond between the spacer backbone and at least one complementing element.

Preferably, step vi) is performed once after every performance of step iv) or once after every performance of step b) or d) of the above mentioned methods.

Accordingly, it is preferred that the covalent bond between the spacer backbone and every complementing element is broken, hence the spacer backbone is preferably not coupled covalently to any complementing element.

In another embodiment the methods furthermore comprise the step of

vii)     breaking the covalent bond between the spacer backbone and at least one functional group.

For example 1, such as 2, for example 3, such as 4, for example 5, such as more than 5, for example more than 10, such as more than 20 covalent bonds between the spacer backbone and functional groups may be broken.

Said covalent bond between the spacer backbone and at least one functional group may in preferred embodiments of the invention be selected from the group consisting of cleavable linkers and selectively cleavable linkers.

Preferably, all covalent bonds between the spacer backbone and the functional entities are broken except for one. More preferably, said bonds are broken after the

formation of the templated molecule. Accordingly, the spacer backbone and the templated molecule are preferably only connected to each other via one covalent bond. For example, said one covalent bond may be a selectively cleavable linker.

5       Ribosomes

Ribosomes are capable of catalysing a reaction betweem spacer reactive groups and thereby forming a covalent bond betweeen spacers.

10      Preferably, ribsomes are capable of catalysing a reaction between building blocks according to the invention, such as a reaction between first building block and/or second building blocks, wherein said reaction result in the formation of a covalent bond connecting said building blocks. Preferably, said covalent bond is formed between spacers of the individual building blocks.

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A preferred ribosome according to the invention is capable of catalysing a reaction between t-RNAs charged with spacers, whereby a covalent bond is formed between the spacers of two charged t-RNAs. Even more preferably, a ribosome according to the present invention is capable of catalysing a reaction between t-RNAs charged

20      with a spacer selected from the group consisting of FE-AA, standard amino acids, non-standard amino acids, pseudo-amino acids and precursors thereof, whereby a covalent bond is formed between said spacers.

Most preferably, a ribosome is capable of catalysing a reaction between t-RNAs

25      charged with standard amino acids, whereby a peptide is formed.

The ribosome may be a wild type ribosome derived from for example an animal, for example a mammal, such as a human being, a plant, a fungi, a yeast or a bacterium. In addition a ribosome may be a mutant or a recombinantly modified

30      version of a wild type ribosome. Methods of genetically engineering a ribosome is known to the person skilled in the art.

Preferably, such a mutant or recombinantly modified ribosome comprises essentially the same activity as a wild type ribosome, such as at least 60%, for example at least

70%, such as at least 80%, for example at least 90%, such as at least 95%, for example at least 99% of the activity of a wild type ribosome. Said activity may be determined by assay measuring the rate of synthesis of a polypeptide.

The ribosome may be a wild type ribosome purified from any organism for example an animal, for example a mammal, such as a human being, a plant, a fungi, a yeast or a bacterium. Alternatively, the ribosome may have been produced using recombinant technology.

Templated molecules

In another aspect, the present invention relates to a templated molecule, a plurality of the same or different templated molecules, wherein preferably each of the templated molecules are obtainable by a method for synthesizing templated molecules according to the present invention.

For example the invention relates to a templated molecule covalently linked to the template encoding said templated molecule, wherein said templated molecule comprises at least 3 covalently linked, functional groups, each encoded by a coding element of said template, with the proviso, that the templated molecule is not a standard α-polypeptide.

Furthermore, the invention relates to a plurality of templated molecules, wherein the plurality comprises at least 1000, such as in the range from 1000 to 5000, for example in the range from 5000 to 10,000, such as in the range from 10,000 to 50,000, for example in the range from 50,000 to 100,000 such as in the range from 100,000 to 500,000, for example in the range from 500,000 to 1,000,000 such as in the range from 1,000,000 to 5,000,000, for example in the range from 5,000,000 to 10,000,000 different templated molecules and wherein said templated molecule comprises at least 3 covalently linked, functional groups, each encoded by a coding element of a template, with the proviso, that the templated molecule is not a standard α-polypeptide.

In particular, the plurality of templated molecules may comprise templated molecules, wherein each templated molecule is selected from the group consisting of templated molecules covalently linked to the template encoding said templated molecule.

The amplifiability of the templated molecules in a molecule library provides said library with a unique feature. This unique feature involves e.g. that a vast number of templated molecules can be screened by taking the library through repetitive processes of selection-and-amplification, in a parallel process where the library of molecules is treated as a whole, and where it is not necessary to characterise individual molecules (or even the population of molecules) between selection-and-amplification rounds.

It is possible according to various preferred embodiments of the invention to screen e.g. more than or about $10^3$ different templated molecules, such as more than or about $10^4$ different templated molecules, for example more than or about $10^5$ different templated molecules, such as more than or about $10^6$ different templated molecules, for example more than or about $10^7$ different templated molecules, such as more than or about $10^8$ different templated molecules, for example more than or about $10^9$ different templated molecules, such as more than or about $10^{10}$ different templated molecules, for example more than or about $10^{11}$ different templated molecules, such as more than or about $10^{12}$ different templated molecules, for example more than or about $10^{13}$ different templated molecules, such as more than or about $10^{14}$ different templated molecules, for example more than or about $10^{15}$ different templated molecules, such as more than or about $10^{16}$ different templated molecules, for example more than or about $10^{17}$ different templated molecules, such as more than or about $10^{18}$ different templated molecules.

As one may perform many repetitive rounds of parallel selection and parallel amplification processes, it is possible to enrich only e.g. 100 fold in each round, and still get a very efficient enrichment, of e.g. $10^{14}$ fold over a number of selection-and-amplification rounds (theoretically a $10^{14}$ fold enrichment is obtained after seven rounds each enriching 100 fold). To obtain a similar enrichment of $10^{14}$ fold using a non-amplifiable library, would require screening conditions allowing $10^{14}$ fold enrichment in one "round" - and this is not practically possible using state-of-the-art

screening technologies. The templated molecules and/or the templates can furthermore be bound to a solid or semi-solid support.

## Methods for screening

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In even further aspects the methods of the invention - individually or as a combination - relates to

a method for screening a composition of complexes or templated molecules
10    potentially having a predetermined activity,

a method for assaying the predetermined activity potentially associated with the complexes or the templated molecules,

15    a method for selecting complexes or templated molecules having a predetermined activity,

a method for amplification of the template that templated the synthesis of the templated molecule having, or potentially having a predetermined activity, and

20

a method for amplification of the template that templated the synthesis of the templated molecule having, or potentially having, a predetermined activity, said method comprising the further step of increasing the number of copies of the templated molecule.

25

## Preferred embodiments of the invention

In a preferred embodiment of this invention a first building blocks comprising:

30    a) at least one complementing entity comprising at least one recognition group capable of recognising a predetermined coding.

b) at least one functional entity comprising at least one functional group and at least one functional entity reactive group

c) at least one spacer comprising at least one spacer reactive group, wherein the spacer is separating the at least one functional entity from the at least one complementing entity

is a tRNA or "tRNA-like" molecule specifying said complementing entities, said functional entities, said spacers and said spacer reactive groups.

In a preferred aspect of this invention a subset of building blocks each comprising

a) at least one complementing entity comprising at least one recognition group capable of recognising a predetermined coding element, and

b) at least one spacer comprising at least one spacer reactive group.

are tRNA or "tRNA-like" molecules specifying said complementing entities, said spacer and said spacer reactive groups.

In a preferred aspect of this invention the linking of spacer groups are performed by ribosomes or by other entities (e.g. DNA, RNA, proteins or combinations thereof, such as ribozymes) or combination of entities (such as ribozyme/protein complexes) enabling template dependent formation of a covalent bond, but preferably by ribosomes.

In a preferred embodiment of this invention the template (or coding element) is DNA, RNA or modified versions thereof including phosphorothiate DNA -or RNA, 2'-O-methyl RNA or mixed nucleic acid sequences, but preferably RNA.

In a preferred aspect of this invention the template that specified the templated molecule is linked to the templated molecule by any covalent or non-covalent means linking the templated molecule and the template. Such linkages may be accomplished by techniques known to those skilled in the art, and may include SPERT or PROFusion (described above) or equivalent techniques.

In one preferred embodiment, the templated molecule is linked to its template by any of the linkages or methods described in patents US 6,214,553 or US 6,207,446, which are both encorporated herein by reference in their entirety. For example, the templated molecule may be linked to its template using puromycin, for example, the template may be tethered to puromycin, which may become covalently linked to the templated molecule.

Hence, in the template/templated molecule complex according to the invention, the template may be linked to the templated molecule via a puromycin linker.

According to the above statements the preferred embodiment of this invention relates to;

a) translation of RNA or other nucleic acid- or nucleic acid derivative templates by ribosomes or by other entities (e.g. DNA, RNA, proteins or combinations thereof) or combination of entities enabling template-specific formation of covalent bond(s).

b) template complementation involving tRNAs or tRNA-like entities comprising a template complementing element, preferably an anticodon, enabling complementation of a template element, preferably a codon.

c) tRNAs or tRNA-like entities comprising a spacer reactive group or groups enabling covalent coupling between a neighbouring spacers or spacers provided by a neighbouring tRNA or tRNA-like entity or entities according to the interaction to the template.

d) formation of a spacer backbone by formation of a plurality of reactions between spacer reactive groups, a reaction preferably catalysed by a ribosome.

e) tRNAs or tRNA-like entities covalently linked to a spacer reactive group(s) wherein the spacer reactive groups are preferably an acyl and/or an amine.

f) tRNAs and tRNA-like entities where the spacer reactive groups are part of a standard α-amino acid, non-standard amino acid, pseudo-amino acid.

g) tRNAs and tRNA-like entities where a subset of tRNA and tRNA-like entities comprise a functional entity (FE) comprising a functional group and functional entity reactive groups capable of forming a covalent link to a neighbouring functional reactive group(s). Each functional group is linked by a cleavable or selectively cleavable linker to the spacer reactive groups. Preferably the functional entity and the cleavable or selectively cleavable linker separating the functional entity and complementing element is an R-side-group of a *i*) standard α-amino acid, *ii*) non-standard amino acid or *iii*) pseudo-amino acid.

h) formation of a spacer backbone which is preferably a standard α-peptide backbone, non-standard- or pseudo-peptide backbone.

i) formation of a spacer backbone comprising two or more functional entities each comprising one or more functional entity reactive group(s) capable of linking by reaction to one or more neighbouring functional entity reactive group(s).

j) coupling of neighbouring functional reactive groups and the formation of a templated molecule of linked functional groups with each functional group linked to atleast one neighbouring functional groups and each functional group further linked to the spacer backbone through the cleavable or selectively linkers.

k) cleavage of none, one or more of the of cleavable linkers, leaving one or more, preferably one, selectively cleavable linker intact, enabling preferably a single covalent coupling between the assembled functionalities of functionalities and the spacer backbone.

l) formation of a covalent or non-covalent link between the templated molecule and the template that templated the templated molecule, preferably the link is via the spacer backbone.

Accordingly, a preferred aspect of this invention relates to the incorporation in the process of translation of non-standard amino acids, comprising or linked to functional entities comprising functional entity reactive group(s), into a peptide comprising an amide- or other type of bonds, but preferably an α-peptide. The non-standard or pseudo amino acids comprising or linked to said functional entities,

hereafter termed FE-AA (functional entity-amino acid) are incorporated into a peptide by ribosomes using tRNA or tRNA-like entities for template decoding where the template preferably constitute an RNA sequence or a modified RNA sequence containing at least one translational start site, preferably AUG and a coding

5    sequence capable of being decoded by said tRNAs or tRNA-like entities.

A FE-AA is attached to a specific tRNA or tRNA-like entity that specifically decode a coding element or a set of coding elements in the RNA template by the interaction between the codon of the RNA template and the anticodon of said tRNA or tRNA-

10    like entity during translation. Preferably, the FE-AA is attached to its cognate tRNA or tRNA-like entity by an acyl-linker similar to that observed for charging of tRNAs by standard amino acids for the purpose of translation. An example of a tRNA charged with an amino acid and the corresponding charging with an FE-AA is shown in figure 4B and 4A, respectively. A few examples of tRNAs charged with FE-AA units are

15    shown in figure 4C. Since individual tRNAs or tRNA-like entities are selectively charged with individual specific FE-AA units and is capable of decoding a specific codon of the template it is possible to incorporate a plethora of FE-AA units into a peptide sequence at predetermined positions according to the template sequence.

20    Charging of individual tRNAs with cognate FE-AA units or when desired with standard-, non-standard or pseudo-amino acids can be accomplished by at least two protocols known to those persons skilled in the art;

i) Enzymatic charging of tRNAs using wt or engineered versions of amino acyl-tRNA

25    synthetases capable of linking by formation of a covalent bond, a specific tRNA and a specific FE-AA unit, standard-, non-standard- or pseudo-amino acid. The linking may be any covalent bond, but preferably an acyl bond between a 2' or 3' OH-group of the tRNA 3'-terminal adenosine nucleotide and a carboxyl group of said, FE-AA unit, standard-, non-standard or pseudo-amino-acids. See figure 5A

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ii) Chemical aminoacylation of tRNAs. Here, each specific in vitro synthesised tRNA missing the 3'-terminal pCpA dinucleotide is ligated by an enzymatic reaction to a pdCpA-dinucleotide chemically charged with a FE-AA unit, standard-, non-standard or pseudo-amino acid. The linking may be any covalent bond, but preferably an acyl

35    bond between a 2' or 3' OH-group of the tRNA 3'-terminal nucleotide and a carboxyl

group (or activated ester) of said, FE-AA unit, standard-, non-standard or pseudo-amino acid. See Figure 5B.

Each peptide should contain atleast 2 FE-AA for example 3 FE-AA, such as atleast 4 FE-AA, for example 5 FE-AA, such as atleast 8 FE-AA, for example 10 FE-AA, such as atleast 15 FE-AA, for example 20 FE-AA, such as 30 FE-AA, for example 50 FE-AA, such as 100 FE-AA, for example 200 FE-AA, such as atleast 300 FE-AA for example 500 FE-AA. It may be desirable that a peptide contain standard, non-standard or pseudo-aminoacids that does not constitute an FE-AA unit. Such non-FE-AA units are preferably not a part of a templated molecule but may serve structural purposes necessary for ordered polymerisation/bond formation required for the formation of the templated molecule according to the predetermined template sequence as described below.

Evidently, a preferred embodiment of this invention dictates that a translational apparatus should be capable of sustaining the conversion of a predetermined template sequence into a standard peptide, non-peptide or pseudo-peptide sequence comprising sidegroups which are not confined to the 20 amino acid sidegroups found in naturally occuring peptides. Several experiments have shown using synthetically engineered tRNAs carrying non-standard amino acid units that both eukaryotic and prokaryotic translation machineries can incorporate said non-standard amino acid or pseudo amino acids in a peptide chain. Consequently, a translational machinery is capable of incorporation non-standard amino acid or pseudo-amino acids such as many L-aminoacids comprising unusual sidegroups, alpha-alpha-disubstituted aminoacids, alpha-hydroxy acids such as lactic-, glucolic- or phenyllactic acids, mercapto acids or N-methyl-aminoacids, conformationally restricted amino acids, amino acids with photoaffinity labels, spin labels and unusual steric properties etc. An incomprehensive list of molecules which have been shown to be incorporated into peptides by in vivo, in situ or in vitro translation by cells, tissues, exstracts or purified components of a translational machinery of eukaryotic or prokaryotic origin as shown in figure 3. The vast chemical and structural diversity of the molecules capable of being incorporated in peptides suggests that the translation process exhibit only a limited discrimination and further argues that most alpha-aminoacids sidegroups including desirable FE sidegroups can easily be incorporated into peptides by ribosomes. The sizes and functionalities of FE-AA

units that are to be incorporated by ribosome mediated translation according to this invention are similar to the sizes and functionalities listed in figure 3.

It may be desirable that translation is conducted by purified reconstituted translation components such as PURE (Protein synthesis using recombinant elements; Shimizu et al., 2001). Translation using purified components offers several advantages;

i) Each specific tRNA capable of recognising a specific codon can be charged with any FE-AA unit, standard-, non-standard or pseudo-amino acid without limitations.

ii) tRNAs charged with FE-AA units, non-standard or pseudo-aminoacids are not deacylated by aminoacyl-tRNA synthetases.

iii) release factors (RF1, RF2 and RF3) can be omitted from the translation reaction preventing premature translation arrest by stop sites and further expands the number of codons by allowing tRNA reading of the UAG, UAA and UGA codons.

iv) reduced level of background "noise" (i.e. irrelevant protein, RNA and DNA).

v) the absence of ribonucleases will increase RNA stability and promote template recovery.

In one aspect of this invention release factors (RF1-3) are omitted from the translation reaction which allows specifically engineered tRNAs to decode the template codons UAG, UGA and UAA normally specifying a translational stop-site. This expands the codon-set and furthermore prevent premature translation-arrest as well as increases the efficiency of template-peptide coupling (when employing PROFusion or similar coupling technology). However, in some aspects of this invention, one or more release factors may be included in a translation reaction.

An FE-AA unit is incorporated into the spacer backbone according to the template sequence. However, the subsequent polymerisation of FE-units is based on a reaction between a reactive group(s) of an FE-unit with a reactive group(s) of an adjacent FE-unit and, thus, governed by a proximity effect. Consequently, in one embodiment of this invention functional groups specified by the template are

assembled into the templated molecule in random order resulting in sequences of functional groups that may not correspond to the order specified by the template sequence.

5      In a further aspect it may be desirable to incorporate an FE-AA unit comprising a scaffolding molecule (see f. ex Figure 4-18) for attaching functional groups producing a templated molecule comprised of the scaffolding molecule and functional groups specified by the template sequence and assembled on the scaffolding molecule at random.

10

Ultimately, the selected template sequences specifying a limited number of functional entities assembled at random allows for the synthesis of each molecule candidate individually for further examination.

15     In a preferred aspect of this invention the assembly of functional groups forming the templated molecule occurs in a non-random and predetermined order. In some embodiments the spacer backbone may adopt predetermined 3-dimensional structures that enable the ordered assembly of functional groups by reactions between FE-reactive groups positioned adjacently according to the 3-dimensional

20     structure. Such structures are formed either concomitantly with or after synthesis of the spacer backbone. In a preferred aspect such structures may be specified by spacer residues that are *not* FE-units. In one aspect the spacer backbone may adopt the form of simple structures such as an $\alpha$-helix, parallel or anti-parallel $\beta$-sheet or $\beta$-turn. However, other higher order structures may include helix-turn-helix

25     and zinc-finger structures, left and right-handed di-, tri-, tetra- or penta-meric coiled-coils, cystine knots, $3_{10}$-helices and parallel $\beta$-helix as well as structures obtainable by interactions with various supports such as a glass-, plastic- and mineral-surfaces, interaction with nucleic acids, solvents and ice-matrices or structure stabilization by magnetic fields. A few relevant structures and their application in the

30     assembly of templated molecules are described below.

$\alpha$-helix: The $\alpha$-helix is a coiled, mainly right-handed, structure present in nearly all natural proteins. The right-handed $\alpha$-helix is stabilised by an array of intra-strand hydrogen-bonds. Some amino acids side-groups such as alanine, glutamate,

35     leucine, isoleucine have a preference for forming an $\alpha$-helix. Since the helix coil

consists of ~ 3.6 amino acid residues per helix turn the side-chains may alter between charged and hydrophobic residues with a periodicity of three or four amino acids creating surface patches with defined properties along the helical axis (Figure 7A). These properties may define helix solubility, stability or the propensity for interaction with other helices to form coiled-coil superstructures as described below. In one aspect of this invention standard, non-standard or pseudo-amino acids are incorporated into a spacer backbone at positions predetermined by the template sequence intended for α-helix formation. FE-AA units are incorporate into said spacer backbone at positions specified by the template preferably at a periodicity of three or four amino acids forming a patch of FE-units arranged at the same face of the helix specified by the spacer backbone (see Figure 7B). Thus, on average an FE-AA unit is incorporated once per helix turn resulting in distance of ~ 6 Å between neighbouring FE-AA units in the helical array. Incorporation of one FE-AA unit per two helical turns results in a distance of ~11 Å between adjacent FE-units. Incorporation of one FE-AA units per three helical turns results in a distance of ~17 Å between adjacent FE-units. Incorporation of one FE-AA unit per four helical turns results in a distance of ~ 22 Å between adjacent FE-units etc. Yet, in a preferred aspect of this invention the FE-AA units are incorporated into the spacer backbone once per helical turn. Consequently, each FE-unit is positioned in close proximity of a neighbouring FE-unit(s) for optimal reaction between adjacent FE-reactive groups enabling efficient FE polymerization and the ordered assembly of the templated molecule according to the template sequence.

Even though some amino acid residues have the propensity for α-helix formation, the single α-helical structure is generally considered unstable and the predictability of such helical structure unreliable. In some cases it may be beneficial to attach negatively charged entities at the C-terminal and/or a positively charged entity at the N-terminal residue of the spacer backbone as dipole-compensating residues augmenting helix stability. In yet another aspect it may be advantageous to apply magnetic field for helix orientation and stabilization according to the helix dipole-moment. In another aspect a putative α-helical structure may be stabilised by solvents or by interactions with polymers such as polyethylene-glycol, poly-phosphate or poly-sialyl via interactions between negatively or positively charged patches of the helix and the counter-ions of the polymer. In a further aspect uncharged or hydrophobic residues of an α-helix may increase helix stability by

interactions with uncharged or hydrophobic units in a polymer. Furthermore, a putative α-helix may be stabilised by interaction with biomolecules such double-stranded DNA or RNA where the imidazole ring of histidine, or the charged amines of lysine and arginine arrayed into patches on the helix can facilitate non-specific interaction with DNA or RNA backbone phosphate-groups, preferably in the minor-groove. Accordingly, the template sequence could be designed such that the spacer backbone conforms to an amphipathic α-helix where FE-units constitute one face of the helix whereas the opposite face comprises residues required for α-helix stability and/or interaction with a helix organising entity. In this set-up the polymerization between FE-units arrayed along the helical axis is initiated after helix stabilization which allows directional polymerisation of FE-units according to the template sequence. Subsequent activation of the templated molecule by cleavage of one or more cleavable linkers is independent of helix stability.

In a preferred aspect of this invention the spacer backbone is an α-helix stabilised by inter- or intramolecular interaction with one or more α-helices forming coiled-coil structure(s).

Coiled-coils: Coiled coils are a bundle of α-helical coils wound into a superhelix (see Figure 8). All coiled-coils have a distinctive repetitive sequence of hydrophobic and hydrophilic residues – the heptad repeat. Each α-helix contains 3.5 residues per turn so that the positions of every seventh residue are eclipsed on the helical surface (i.e. occupy the same radial position when viewed on the helical wheel). In most natural and designed coiled-coils leucine residues constitute the hydrophobic core involved in inter-helical pairing resulting in dimers, trimers, tetramers or pentamers dependent on the level of helix hydrophobicity. Coiled-coils may be parallel or anti-parallel and contain any of several possible heptad repetitive sequences comprising interchanging hydrophobic and hydrophilic residues according to the consensus HPPHPPH, HPPPHPP or HPPHPPP (H = hydrophobic residues, P = polar residues). A subset of coiled-coils are leucine zippers comprising repetitive heptad sequences of the consensus LXXYXXX or YXXLXXX where L denotes leucine, Y is leucine, isoleucine or valine and X any amino acid. The structures may be stabilised further by oppositely charged residues positioned in register on opposing helices resulting in electrostatic interactions referred to as acid-base coiled-coils (see Figure 8). Further coiled-coil stabilising elements may include cysteine residues positioned

at helix ends forming inter-helical disulfide bonds or β-lactam units increasing core hydrophobicity.

FE-AA units can be incorporated into a spacer backbone predisposed for coiled-coil formation. Furthermore, the FE-AA units can be incorporated into a spacer backbone according to the template sequence positioned opposite the hydrophobic core involved in dimerisation and thus exposed into solution. In a preferred aspect of this invention it is possible to incorporate FE-AA units once per helical turn, once per two helical turns, once per three helical turns or once per four helical turns etc, but preferably once per helical turn within the repetitive helical segments of the coiled-coil structure. A few examples of repetitive heptad sequences designed for dimer coiled-coil structure formation are listed below.

$$M\text{-}C\text{-}X_n\text{-}(L\text{-}K\text{-}U\text{-}E\text{-}Y\text{-}P\text{-}U)_n\text{-}X_n\text{-}C\text{-}X$$
$$M\text{-}C\text{-}X_n\text{-}(L\text{-}E\text{-}U\text{-}K\text{-}Y\text{-}P\text{-}U)_n\text{-}X_n\text{-}C\text{-}X$$
$$M\text{-}C\text{-}X_n\text{-}(L\text{-}K\text{-}U\text{-}E\text{-}Y\text{-}U\text{-}P)_n\text{-}X_n\text{-}C\text{-}X$$
$$M\text{-}C\text{-}X_n\text{-}(L\text{-}E\text{-}U\text{-}K\text{-}Y\text{-}U\text{-}P)_n\text{-}X_n\text{-}C\text{-}X$$

M = Methionine, C = Cysteine, P = polar residue, U = FE-unit.

Insertion of cysteines for di-sulfide bridge formation or E and K for inter-strand ionic interactions are optional but may be beneficial in cases where the FE-polymerisation reaction is conducted in adverse conditions (high temperature, high pH, in solvents etc.).

Coiled-coil formation requires that the spacer backbone comprising the FE-units should dimerise with a second helical element predisposed for coiled-coil structure formation. Thus, the spacer backbone comprising FE-units may dimerise with another spacer backbone comprising FE-units or with a helical element that does not contain FE-units.

Peptide strands that does not contain FE-units may be produced separately by solid-phase chemical synthesis

In another aspect it is possible that a spacer backbone comprising FE-units contain two helical elements each predisposed for coiled-coil formation separated by a turn in the spacer backbone structure resulting in an intra-molecular coiled-coil.

In yet another aspect of this invention it is possible to form trimers, tetramers or pentamers by increasing the number of hydrophobic residues. The use of higher order coiled-coil structures may be useful for the polymerisation of displayed FE-units in solvents, at high pH or at high temperatures.

Collagen triple-helix: Collagen is a highly stable triple helix structure where each peptide strand contains repetitive units of the sequence;

Glycin-Proline–X ,

where, X denotes any aminoacid, preferably a polar residue. The single backbone strand contains ~ 3 residues per turn with a helical rise of 2.9 Å. Consequently, it is possible to incorporate FE-AA units into a spacer backbone comprising the following repetitive trimeric sequence;

(Glycine-Proline-U)$_n$ , U = FE-unit.

After formation of a triple helix super-structure each FE-unit will be displayed into solution. The average distance between neighbouring FE-units will be ~ 9Å. Each strand displaying FE-unit may associate with one or two other strands containing FE-units or with one or two strands not containing FE-units. Peptide strands not containing FE-units may be produced separately by solid-phase chemical synthesis.

It may be advantageous to use a collagen triple-helix structure for FE-display in the cases where the polymerisation reaction is conducted under conditions not easily compatible with protein secondary structure.

β-helix: This structure forms multiple parallel sheeted folds forming a tube structure. Sheet residues either protrude into the largely hydrophobic interior of the tube or outwards into solution and adjacent residues on the same face of neighbouring

sheets are separated by less than 5 Å. Thus, in one aspect it is possible to incorporate FE-AA units displayed in either a hydrophobic or a polar environment. Furthermore, the close proximity of neighbouring FE-units should benefit the overall polymerisation efficiency, thus increasing the production of templated molecules.

In a preferred aspect of this invention the FE-display structures described above require that only a limited number of the total codon-set is used for incorporation of standard-, non-standard- or pseudo-amino acids (i. e. non-FE-units). Preferably, less than 5 codons are used for the incorporation of non-FE-units according to the invention

Thus, the present invention disclose a method for templating a templated molecule comprising FE-units specified by the template sequence.

Provided that complementation of neighbouring coding elements is achieved, neighbouring, spacer reactive groups of a building block are capable of being covalently linked forming a spacer backbone. The random or non-random display of FE-units enable reactions between FE-reactive groups of neighbouring FE-units forming a polymer or a branched molecule of FE-units specified by the template sequence. In a further application it is possible subsequently to maintain or cleave the cleavable linker separating the functional entity from the complementing element defining said functional entity without cleaving the link between neighbouring functional groups of a templated molecule.

Also disclosed are methods for identifying the sequence and/or identity of functional groups of a templated molecule, as well as methods for therapy and diagnostic methods exploiting the templated molecules according to the invention.

In yet another aspect there is provided a method for altering the sequence of a templated molecule, including generating a templated molecule comprising a novel or altered sequence of functional groups, wherein the method comprises the step of mutating the template that templated the synthesis of the original templated molecule. The method preferably comprises the steps of

providing a first template capable of templating the first templated molecule, or a plurality of such templates capable of templating a plurality of first templated molecules,

5    modifying the sequence of the first template, or the plurality or first templates, and generating a second template, or a plurality of second templates,

wherein said second template(s) is capable of templating the synthesis of a second templated molecule, or a plurality of second templated molecules,

10

wherein said second templated molecule(s) comprises a sequence of covalently linked, functional groups that is not identical to the sequence of functional groups of the first templated molecule(s), and optionally

15    templating by means of said second template(s) a second templated molecule, or a plurality of such second templated molecules.

The above-mentioned method exploits that a templated synthesis in one embodiment involves a single-stranded, modifiable intermediate in the form of a

20    template. In the case where this template comprises a nucleotide strand comprising deoxyribonucleotides or ribonucleotides, most molecular biological methods can be applied to modify the template, and therefore to modify the templated molecule.

The present invention also relates to building blocks used for synthesising the

25    templated molecule and to complexes comprising such building blocks. In another aspect there is provided the use of a building block for the synthesis of a templated molecule according to the invention. In a preferred embodiment of this aspect, the templated molecule comprises or essentially consists of a molecular entity capable of binding to another molecular entity in the form of a target molecular entity or a

30    binding partner.

The templated molecule is preferably a medicament capable of being administered in a pharmaceutically effective amount in a pharmaceutical composition to an individual and treating a clinical condition in said individual in need of such

35    treatment.

In other aspects of the invention there are provided a pesticidal composition, an insecticidal or herbicidal composition, a bacteriocidal or bacteriostatic composition, and a fungicidal composition, as well as methods for preparing such compositions and uses thereof, wherein each of said compositions comprise a templated molecule according to the invention in an amount effective to achieve a desired effect.

In still further aspects there is provided a method for identifying a pharmaceutical agent, or a diagnostic agent, wherein said method comprises the step of screening a plurality of drug targets with at least one predetermined, templated molecule, and identifying a pharmaceutical agent, or a diagnostic agent, in the form of candidate templated molecules capable of interacting with said drug targets.

In yet another aspect there is provided a method for identifying a target, including a drug target, wherein said method comprises the step of screening a plurality of ligands or receptor moieties with at least one predetermined, templated molecule, and identifying drug targets in the form of ligands or receptor moieties capable of interacting with said templated molecules.

The present invention also relates to any isolated or purified templated molecule having an affinity for a predetermined target, including a drug target, as well as to targets, including drug targets, in the form of ligands, receptor moieties, enzymes, cell surfaces, solid or semi-solid surfaces, as well as any other physical or molecular entity or surface having an affinity for a predetermined templated molecule.

In even further aspects of the invention there is provided a method for treatment of an individual in need thereof, said method comprises the step of administering to the individual a pharmaceutically effective amount of a molecule identified by a method of the present invention and having an affinity for a predetermined target, including a drug target.

In a still further aspect there is provided a method for treatment of an individual in need thereof, said method comprises the step of administering to the individual a pharmaceutically effective amount of an isolated or purified ligand or receptor moiety

having an affinity for a predetermined templated molecule according to the invention. The isolated or purified ligand or receptor moiety is preferably identified by the above-mentioned method of identification of the invention.

5

**Examples**

The following example illustrates specific embodiments of the invention and should not be regarded as limiting for the invention.

10

**Example of synthesis, selection and amplification of encoded β-peptide molecules capable of binding to a receptor protein.**

Below is described the synthesis of a library of molecules capable of being tested for
15     desired characteristics. Molecules with relevant properties can be selected and their templates amplified allowing enrichment of templates encoding said molecules. Multiple rounds of molecule selection and amplification of co-selected templates enable the isolation of molecules with unique properties.

20     A DNA template is provided encoding a T7-RNA promoter sequence, a translational initiation element comprising a Shine&Dalgarno and an ATG codon followed by a random sequence of 18 nucleotides and a fixed sequence of 16 nucleotides with the sequence; 5'-TAGTCCGAATCCCGGG-3'. Said template is transcribed producing at least $10^{13}$ different RNA molecules according to a standard procedure with the
25     following composition: 100 mM Tris-HCl, 22 mM MgCl$_2$, 4 mM each of UTP, CTP, ATP, GTP, 10 mM DTT, $10^{13}$ DNA different template molecules, 1u/μl RNasin, and 1u/μl T7 RNA polymerase and incubated at 37°C for 4h.
Following RNA synthesis the DNA template is removed by addition of 10 units of DNase I. The RNA template molecules are recovered through gel-filtration or by
30     preparative gel-electrophoresis according to standard procedures.

Purified RNA is tagged by DNA-puromycin entity at the 3'-end according to the method shown in figure 2 (Roberts et al.1997). In brief, a fixed DNA sequence dA27dCdC- followed by a 3'-terminal puromycin residue is attached to the RNA 3'-
35     end by ligation using T4 RNA ligase and a DNA oligonucleotide that enhance the

ligation efficiency. Following addition of the DNA-puromycin-tag the RNA-DNA-puromycin chimeric molecules are purified by gel-filtration or preparative gel-electrophoresis.

5      The purified and tagged RNA fragments are in vitro translated using standard in vitro translation or preferably by purified components (PURE, Shimizu et al., 2001) and tRNAs charged with FE-AA units, standard, non-standard or pseudo-amino acids. The tRNAs to be charged are synthesised from plasmid preparations carrying a specific anticodon triplet sequence but missing the 3'-end CpA dinucleotide in a

10     tRNA synthesis mixture with the following composition:100 mM Tris-HCl, 22 mM $MgCl_2$, 4 mM each of UTP, CTP, ATP, GTP, 10 mM DTT, 2 mM spermidine, 200 µg of DNA template molecules, 1u/µl RNasin, and 1u/µl T7 RNA polymerase in total volume of 1 ml and incubated at 37°C for 4h. The above synthesis is conducted for 64 different plasmid templates each encoding a tRNA sequence containing a unique

15     anticodon triplet sequence. Subsequently, the pre-tRNAs (missing the CpA dinucleotide) is purified using gel-filtration, gel-electrophoresis or preparative HPLC.

A pdCpA dinucleotide is purchased or synthesised by standard phosphoamidite chemistry described in Robertson et al., 1989, 1991.

20

Prior to the charging of the tRNAs, pdCpA dinucleotides are chemically acylated using protected FE-AA, amino acids, non-amino acids or pseudo-amino acids. Preferably, the cyanomethyl esters of FE-AA, amino acids, non-natural amino acids or pseudo-amino acids are used for the selective mono-acylation of 3' or 2' hydroxyl

25     groups of the adenosine of the pdCpA dinucleotide. Protection of sensitive amino groups of FE-AA, natural-, nonnatural- and pseudo-amino acids can be accomplished by formation of their nitroveratryloxy (NVOC) carbamate, ester or ether derivatives as described elsewhere (Robertson et al., 1991). Alternatively, the biphenylisopropyloxycarbonyl (BPOC) protective group have been used (Robertson

30     et al., 1991). These protective groups can be removed at any convenient step prior to translation by photoactivation or mildly acid conditions, respectively (Robertson et al., 1991; Mendel et al., 1995). Here, initial amino-protection is by t-butyl (Boc) carbamate formed by addition of $(Boc)_2$ and $H_2CO_3$ for 30 min at room temperature. Following protection, solid calcium phosphate is added and the product extracted by

35     ethylacetate.

Formation of cyanomethylester derivatives of FE-AA, amino acid, non-natural— and pseudo-amino acid for the pdCpA acylation step is accomplished by standard chemistry f.ex using DCC (dicyclocarbodiimide) and hydroxyacetonitrile.

5

Coupling of the cyanomethylesters and the pdCpA dinucleotide is accomplished by reaction between the cyanomethyl ester derivative and the tetrabutyl ammonium salt of pdCpA in DMF and triethylamin at 50 °C for 2 hours (Modified from the protocol of Robertson et al., 1989, 1991). The Boc-amino protective groups is removed by

10    addition of $CCl_3COOH$ to the mixture and reacted for another hour. The charged and amino deprotected acylated dinucleotide is purified using preparative HPLC.

.Each individual dinucleotide charged with a specific FE-AA, natural-, nonnatural- or pseudo-amino acid is coupled to a specific pre-tRNA using T4 RNA ligase in a

15    reaction of the following composition: 42 mM HEPES-KOH, pH 7.4, 10 DMSO, 4 mM DTT, 20 mM $MgCl_2$, 0.2 mg/ml bovine serum albumin, 150 μM ATP, 10 % PEG6000, 2,000 units T4 RNA ligase. The reaction is incubated at 37 °C for 10 minutes and quenched by addition of 1/10 volume 3M Na-acetate (pH 4.5). The mixture is extracted with equal volume phenol/chloroform/isoamylalcohol (25:24:1,

20    pH 4.5) and once with chloroform/isoamylalcohol (24:1) followed by ethanol precipitation and lyophilized. The charged tRNA is resuspended in 2 mM Na-acetate pH 4.5 and stored at -80 °C. Example of a complete tRNA charging protocol is shown in figure 26.

25    Figure 27 shows the individual charged tRNAs. In vitro translation using PURE (Shimizu, 2001) requires the purification of coupled ribosomes. Coupled ribosomes are purified from *E. coli* strain MRE600 grown in LB-broth at to an OD450 of 0.5. Cells are snap-cooled on ice and harvested. The cell pellet is resuspended in 10 mM Tris-HCl pH 7.5, 10 mM Mg-acetate, 100 mM ammonium acetate and 6 mM

30    mercaptoethanol. 10 mg/ml of lysozyme is added and the sample is snap-frozen and thawed on ice (3 times). Insoluble particles are pelleted by centrifugation and the supernatant is loaded onto 5-20 % sucrose gradients in a ribosome buffer: 20 mM Tris-HCl, pH7.5, 15 mM Mg-acetate, 100 mM ammonium acetate. Gradients are ultracentrifuged at 28.000 rpm for 14 hours and the ribosome profile in the gradient

35    is monitored by UV-absorbance. Fractions containing 70S ribosomes are pooled

and collected by ultracentrifugation. The purified coupled ribosomes are resuspended in ribosome buffer and stored at – 80 °C.

Additional proteins and enzymes required for PURE translation such as translation initiation factors 1-3, elongation factors G, Tu Ts, release Factors 1-3, ribosome recycling factor, methionineformyl transferase are purified as His-tagged versions from *E.coli* according to the procedure described by Shimizu et al., 2001. Other enzymes and reagents are available from commercial sources.

The constituents of a PURE translation mixture is shown below (Shimizu et al., 2001)

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Coupled ribosomes (here, from *E. coli*)

Initiation factors 1,2,3

Elongation factors G, Tu, Ts

Ribosome recycling factor (RRF)

10 mM Mg-acetate

5 mM K-phosphate pH 7.3

95 mM K-glutamate

5 mM ammonium-chloride

0.5 mM Calcium-chloride

1 mM Spermidine

8 mM putrescine

1 mM dithiothreitol (DTT)

2 mM ATP

2 mM GTP

10 mM creatine phosphate

0.5 µg 10 formyl-5,6,7,8-tetrahydrofolic acid

0.2 µg creatine kinase

0.15 µg myokinase

0.06 µg nucleoside-diphosphate kinase

0.1 unit pyrophosphatase

Enzymatically or chemically charged tRNAs

RNA template (DNA-puromycin tagged)

PURE translation mixture is incubated at 37°C for 1h.

5　　Following translation, buffer, salts, nucleotides and other low molecular weight components are removed by gelfiltration. Furthermore, this step removes any translation products that are not coupled to their template sequence via the puromycin-linker.

10　　Complexes consisting of translation products fused to their template is purified using poly(dT)-sepharose (AmershamPharmacia) according to established protocols. In brief, the above reaction mixture is incubated with 1 milligram of poly(dT)-sepharose for complex binding via the poly(dA)-tail of the template in binding buffer [$TEN_{200}$, 10 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, pH 7.5] at 4 °C. Multiple washing steps

15　　using binding buffer followed by elution using high salt buffer [$TEN_{2000}$; 10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl] allows for the purification of translation products fused to their corresponding template. Excess salt is removed by gel-filtration.

　　The single stranded mRNA templates are converted to doublestranded RNA/DNA

20　　duplexes by the synthesis of cDNA. cDNA synthesis is conducted by annealing an oligodeoxynucleotide of the following composition; 5'-GG27T-3', to its complementary sequence of the 3' DNA linker portion on the mRNA template, incubated for 1 hour at 42 °C in a reaction mixture of the following composition: 50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine,

25　　200µM each of dATP, dGTP, dCTP and dTTP. Buffers and low molecular weight reagents are removed by gel-filtration.

　　The functional entities (see figure 27) comprised within the translation product each contain reactive groups capable of being reacted with a reactive group of an

30　　adjacent or neighbouring functional entity or capable of reacting with a EDC/NHS to form a coupling between neighbouring carboxylic acids and amines producing a β-peptide molecule. The reaction is carried out in a reaction mixture of the following composition; 20 mM Na-borate pH 9.0, 50 mM KCl, 100 mM EDC and 10 mM NHS and reacted > 2 hours at 40 °C. Buffer and unreacted EDC/NHS is removed by

gelfiltration. The reaction scheme for bond formation between reactive functional groups is shown in figure 28.

Following bond formation between carboxylic acid and amine reactive groups an
5 activation step is employed to eliminate multiple linkages between the functional entities and the peptide (spacer) backbone leaving a single linker intact. Here, the nitrophenyl linkages are cleaved by UV-irradiation at > 300 nM for 15 min at 4 °C using a xenon-lamp and polystyrene plastic protection as filter. This final activation step produce a library of templated molecules each linked to their template/cDNA
10 sequence.

The library of templated molecules is employed for the selection of ligands having desired properties. Here, we are interested in selecting ligands capable of binding gamma-aminobutyric acid (GABA) A receptor, subtype α, a receptor family involved
15 in facilitating sedation, amnesia and seizure protection etc. (Mohler et al., 2002). The receptor protein is obtained from a recombinant source by cloning the gene for GABA receptor A, subtype α, in the bacterial expression system pET as a non-fusion variant (Novagen, Inc). The receptor protein is expressed and purified from *E. coli* strain BL21 using established protocols. The purified recombinant GABA
20 receptor protein is attached covalently to a solid support matrix by chemical coupling between CNBr-activated sepharose 4B (AmershamPharmacia) and exposed amino groups on the receptor protein.

The GABA receptor A, subtype α, attached to the sepharose 4B is used as target in
25 the selection of ligands from the library of templated molecules. To eliminate ligands that bind the matrix a counterselection step is employed. In brief, the pool of templated molecules are poured onto a 50 ml column containing 10 ml of settled CNBr-sepharose 4B in a binding buffer [25 mM Tris-HCl (pH 7.5), 150 mM K-glutamate, 5 mM MgCl₂, 5 mM DTT]. Following elution, 20 ml of binding buffer is
30 added and the eluent is collected. The eluent is loaded onto the settled sepharose 4 B matrix displaying the linked recombinant GABA receptor A, subtype α. Following succesive washing steps using binding buffer, ligands are eluted using using 10 ml of elution buffer [25 mM Tris-HCl (pH 7.5), 2 M NaCl, 5 mM DTT]. Salt and low molecular weight reagents are removed by gelfiltration followed by concentration
35 using lyophilization. The ligands comprising templated molecules linked to their

templates are resuspended in low salt buffer [50 mM Na-acetate (pH 4.5)]. 50 units of chymotrypsin (Roche) are added to remove remaining peptide (spacer) backbone attached to the template sequence and the mixture is incubated for 15 minutes at 50 °C. The reaction mixture is extracted twice with an equal volume of phenol (pH 6.5)

5 followed by extraction with an equal volume of chloroform and finally precipitated using ethanol.

The precipitated templates are amplified by PCR to generate doublestranded DNA templates for transcription. The PCR reaction is conducted using the oligo

10 deoxynucleotide (T7) 5'-CCGGGATCCTG<u>TAATACGACTCACTATA</u>GGCTGATCGATTTCAGTACGGAGG-3' and (PR) 5'-CCCGGGATTCGGACTA-3' in a reaction mixture of the following composition: 50 mM Tris-HCl (pH 7.8), 50 mM KCl, 250 µM each of dGT, dATP, dCTP and dTTP, 2 mM $MgCl_2$, and isolated templates. Following PCR, buffers and

15 unincorporated deoxynucleotides are removed by gelfiltration before synthesis of RNA templates as described above. The RNA templates are used for a second cycle of tagging and translation followed by cDNA synthesis, polymerisation of functional entities, activation of the encoded molecules, counterselection and selection of ligands and finally PCR amplification of templates encoding selected

20 ligands. This scheme is iterated until a limited number of different template sequences are represented in the selected pool (usually less than 1000 different candidates). In most cases 8-12 cycles will suffice.

Finally, templates sequences isolated after 8-12 amplification/selection cycles are

25 identified by cloning and sequencing enabling the identification of the chemical composition of selected ligands. These ligand candidates may be further characterised using pharmacokinetic, pharmacodynamic, toxicologic and in vivo behaviour etc.

30

## Description of the Figures

The following symbols are used in the following figures to indicate general characteristics of the system: In figures 10-24, a long horizontal line symbolizes a

spacer backbone. For clarity, in some of the figures only the polymerization step, not the activation step, has been included. Rx denotes functional groups.

**Figure 1A: Templated synthesis of a linear molecule - The principle.**

5 Figure 1A illustrates an example of a protocol for synthesis of a linear templated molecule. The protocol can be divided into 9 steps with the starting material being either DNA or RNA of desired sequence composition. i) RNA template synthesis, ii) addition of 3'-end DNA-puromycin linker iii) Incorporation by translation iv) complementation of RNA template (cDNA), v) polymerization/bond formation, vi)

10 activation, vii) selection/screening, viii) amplification, and ix) characterization. Template-directed RNA synthesis may be mediated by enzymes such as T7, T3 or SP6 RNA polymerase to yield translational competent RNA templates or by chemical synthesis.

A DNA-puromycin tag is added to the 3'-end of each RNA template according to the

15 procedure disclosed by Roberts et al. 1997 which may include ligation using ligase and a DNA oligonucleotide (splint) or by psoralen cross-linking as described by Kurz et al 2000 (se also Figure 2). The puromycin-linker is used in order to facilitate the coupling between the peptide product and the nucleic acid template. Additional protocols for the coupling templated molecules and their templates are described in

20 the "Summary of the invention" section.

In a preferred aspect of this invention the incorporation of building blocks into a spacer backbone according to the template sequence is accomplished by ribosome mediated translation. Upon completion the spacer backbone is attached covalently to its template via the puromycin entity. Each spacer unit or a subset of spacer units

25 incorporated into the spacer backbone specifies functional entities according to the template sequence and comprise one or more functional entity reactive groups and a cleavable or selectively cleavable linker. The template comprises primer binding sites at one or both ends (allowing the amplification of the template). The remaining portion of the template sequence may be random, partly random or fixed. Annealing

30 of a primer to a fixed sequence in the template 3'-end denoted the priming region (PR) allows template complementation by Reverse transcriptase forming cDNA. Reactions between reactive groups of the functional entities are initiated in a step forming covalent bonds between adjacent functional entities. These bonds are separate from those bonds synthesised by the peptidyl transferase activity of the

35 ribosome forming the spacer backbone.

Activation involves cleaving some or all but one of the cleavablelinkers that connect functional entities to the spacer backbone.

Selection or screening involves enriching the population of template-templated molecule pairs for a desired property.

5 Amplification involves producing more of the template-templated molecule pairs, by amplification of the template or complementing template, and producing more of the template-templated molecule pairs, for further rounds of selection/screening. This step does not require characterization of individual sequences but can be performed on the population of molecules. Following multiple rounds of selection/screening,

10 selected template-molecule pairs are sequenced and/or characterised.

Cloning and sequencing involves the cloning of the isolated templates or complementing templates, followed by characterization. In some cases, it may be desirable to sequence the population of isolated templates or complementing templates, wherefore cloning of individual sequences are not required.

15

### Figure 1B. Templated synthesis of a branched molecule.

The templated synthesis of a branched molecule can be produced using a principle similar to that described in the legend to figure 1A. Functional entities are incorporated into a spacer backbone according to the template sequence. The

20 scaffold is attached either covalently or non-covalently to the template that templated the formation of a spacer backbone comprising functional entities. Reactive groups of said functional entities are capable of reacting thereby forming a covalent bond with the scaffold resulting in a templated molecule comprised of functional entities as substituents on a central scaffold. The covalent attachment

25 between reaction between each functional entity and the scaffold may be directional (i.e. each subgroup of functional entities is only capable of reacting with one specific position on the scaffold) or a random process (all functional entities can react with any of the positions on the scaffold).

30 ### Figure 1C: Example of a FE-units and non-FE-units displayed on a spacer backbone connected to its cognate template.

### Figure 2. A method used for retro-genetic tagging of peptides (PROFusion).

Steps A-C are schematic representations of steps involved in the production of RNA-protein fusions. **A**, illustrates a sample DNA construct for generation of a RNA portion of a fusion. **B**, illustrates the generation of an RNA/puromycin conjugate. **C**, illustrates the generation of an RNA-protein fusion.

5

**Figure 3. Examples of non-standard amino acids and pseudo-amino acids capable of being incorporated into peptides.**

Examples of amino acid-like entities known to be incorporated into peptides by in vivo or in vitro translation.

10

**Figure 4: Building blocks:**

A: Example of a first building block comprising a complementing element, a functional entity, a cleavable linker and spacer reactive groups.

B: Example of a second building block comprising a complementing element and
15    spacer-reactive groups. The shown first building block comprises a tRNA charged with FE-AA unit capable of being incorporated into a spacer-backbone by ribosome mediated translation. Subsequent to the synthesis of the spacer-backbone the functional entity can participate in the formation of a templated molecule producing an $\alpha,\beta$ disubstituted $\beta$-peptide.

20    C: Examples of building blocks comprising FE-AA units. Shown is the 3'-terminal adenosine of a tRNA (complementing element) charged with FE-AA units via a covalent bond involving the adenosine 3' or 2' hydroxy-group. Below are listed examples of conditions used for bond formation between neighbouring FE-AA and for cleavage of cleavable or selectively cleavablelinkers. 1) "fill-in" homobifunctional
25    activated esters, cleavage by nucleophile; 2) Carboxyanhydride activated for step-by-step polymerisation by a primary amine, cleavage by nucleophile. 3) "fill-in" by homobifunctional activated esters, photocleavage; 4)  Thiocarboxyanhydride activated for step-by-step polymerisation, cleavage by nucleophile; 5) Coumarin based coupling by photoactivation, cleavage by catalysis ($H_2$/Pd); 6) Double
30    activated phosphoester unit. Coupling using dihydroxylated such as 1.3 dihydroxypyrimidine and enzymatic cleavage of linkers by chymotrypsin. 7) "fill-in" using activated bifunctional esters, photocleavage of linkers; 8) "fill-in" by activated esters, linker cleavage by elevated temperature; 9) Coupling by "fill-in" by ketone-hydrazide reaction and by modified Staudinger, linker cleavage by nucleophile; 10)
35    "fill-in" coupling using double-activated esters, photocleavage of linkers. 11) Direct

coupling using EDC/NHS; 12) "fill-in" coupling using double activated esters, linker cleavage by nucleophile; 13) "fill-in" by diamin and EDC/NHS activation of carboxy groups, linker cleavage by acid treatment; 14) "fill-in" by pericyclic coupling f. ex. By 1.4 benzoquinone, linker cleavage by nucleophile; 15) α,β disubstituted β-amino acid precursor, coupling by ring opening. Activated by a primary amin, linker cleavage by reduction of disulphide bridge (f.ex by DTT addition); 16) β-aminoacid precursor capable of being translocated upon ester reaction with n adjacently positioned ora neighbouring amine of an adjacently positioned or neighbouring FE-AA, thus, coupling and linker cleavage occurs in the same reaction step; 17) Stepwise coupling of carboxyanhydride that are activated by a primary amino group. The "traceless" linker is photcleaved. 18) Scaffold molecule with reactive groups and no cleavable linker.

## Figure 5: Charging of a complementing entity.

A: Enzymatic charging of a complementing entity by aminoacylation using amino acid tRNA synthetases for catalysis. Enzymatic charging may be carried out using wt, modified or mutant aminoacyl-tRNA synthetases. Alternatively other entities capable of charging a complementing entity such as selected or engineered RNA or DNA aptamers may conduct the charging of a complementing element.

B: Chemical charging of a complementing entity. Shown is the chemical charging of a tRNA using a 2-step protocol described by Mendel et al., 1995. A tRNA, lagging the essential 3' proximal CpA dinucleotide (cytidine-phospho-adenosine) is synthesised in vitro using T7 RNA polymerase and a DNA template comprising a promoter for T7 RNA polymerase transcription and the corresponding tRNA sequence (minus 3'-end CpA). A pdCpA dinucleotide is synthesised using phosphoamidite chemistry and charged with an FE-AA entity a carrying convenient protection group(s) such a Boc, NVOC or, Foc.

The charged complementing entityoccurs by water elimination resulting in an ester linkage between the FE-AA entity and the 2' or 3' hydroxyl group of the terminal adenosine residue. The charged pdCpA dinucleotide is ligated to the 3'-end of the pre-tRNA seqment and subsequently deprotected using acid, photocleavage or similar technique depending on the protective group(s). These steps enable the chemical charging of a tRNA comprising a specific complementing element (codon) with a specific (cognate) FE-AA entity or any entity such as aminoacids, non-natural amino acids or pseudoamino acids capable of being incorporated into a spacer-

backbone by ribosome-mediated translation. The first charging step shown involving addition of FE-AA, amino acid, non-natural amino acid or pseudo-amino acid to the pdCpA can be conducted using oligonucleotides other than the pdCpA. The charging can be conducted using mono, tri, tetra, penta, hexa, hepta, octa, nona, deca etc oligonucleotides of appropriate sequence. Thus, the extent of the pre-tRNA to be ligated to the charged oligonucleotide should be designed accordingly.

## Figure 6: Bond formation and Activation

Example of bond formation and activation by cleavage of cleavable linkers, whereina disubstituted β-peptide comprising two monomer units linked to the spacer backbone is formed. After translation of a RNA template the functional entities are displayed on a spacer backbone (shown here at every second position). Bond formation is initiated by deprotection of an amine group, for example by a photoactivation step. Next, the primary amine attacks the carbonyl group of a neighbouring N-thiocarboxy anhydride (NTA) forming an amide bond upon releasing CSO. This reaction produce a primary amine for a second bond formation step involving the neighbouring NTA-unit resulting in the formation of a β-peptide comprising two monomer units and a non-cleavable linker. Subsequently, the β-dipeptide is activated by cleavage of the ester linkages connecting the β-peptide units and the spacer backbone resulting in a β-dipeptide connected to the spacer backbone via a single selectively cleavable linker. The template that encodes the β-dipeptide may be attached to the spacer backbone at any suitable position, but preferably at the C-terminus of the spacer backbone. For simplicity, the template has been omitted from the figure.

## Figure 7: Display of FE-units by incorporation into an α-helix.

A: helical view down the axis of an α-helix. FE-units are displayed on the same face of the helix such as in position 2, 6, 9 etc or preferably as closest neighbour in position 2, 6, 10 etc. according to the helix structure.

B: FE-units incorporated at every fourth position in an α-helix. The template is attached to the spacer backbone at any convenient position (here, at the C-terminus of the spacer backbone). Following bond formation and activation of the linked FE-units the templated molecule is formed. The templated molecule is attached to the spacer backbone by one or more linkers and physically attached to its cognate template via the spacer backbone. Following selection of templated molecules with

desired properties, the appended template is amplified and transcribed by RNA polymerase. Purified RNA is tagged by DNA/puromycin in the RNA 3'-end, re-purified and translated.

5      To prevent interference in the selection procedure it may be advantageous to remove the main portion of the spacer backbone. One method for linker removal is to incorporate a lysine or arginine residue in the spacer backbone N-terminal of the spacer backbone unit connecting the templated molecule and the spacer backbone.

**Figure 8: Display of functional entities by a coiled-coil structure.**

10     Translation of a template will produce a spacer backbone (peptide) comprising FE-units. A subset of spacer backbone units incorporated into the template spacer backbone and which is not an FE-unit predispose the spacer backbone for coiled-coil structure formation. After translation, coiled-coil structure formation is initiated by interaction between two spacer backbones each comprising FE-units or between a

15     single spacer backbone comprising FE-units and a second spacer backbone (peptide) that does not contain FE-units. Such a second spacer-backbone may be prepared separately by solid-phase chemical synthesis, in vitro translation or by purification from cells of any source expressing any desirable spacer backbone capable of coiled-coil formation. Coiled-coil structure formation may also be induced

20     by intramolecular interaction between spacer segments separated by a turn structure. Following coiled-coil structure formation neighbouring FE-units are cross-linked. Subsequent cleavage of a subset of linkers produce a templated molecule linked to its template via at least one spacer unit. The template::templated molecule complexes with desired properties are selected and the templates are amplified

25     followed by transcription producing RNAs for DNA/puromycin tagging and subsequent translation.

**Figure 9: Display of functional entities by a collagen triple-helix structure.**
Translation of a template produce a spacer backbone comprising FE-units and

30     spacer units enabling formation of a triple helix collagen-like structure. Subsequent to translation the triple helix structure is produced by interaction between three spacer backbones comprising FE-units or between one or two spacer backbones comprising FE-units and two or one spacer backbone not comprising FE-units, respectively. Such spacer backbones may be produced by solid-phase chemical

35     synthesis, in vitro translation or by purification from cells of any source expression

desirable spacer backbone molecules. Following triple helix formation FE-units are cross-linked. Cleavage of a subset of linkers produce a templated molecule, preferably connected to the spacer backbone by a single linker.

5     Template::templated molecule complexes with desired characteristics can be selected and their templates amplified enabling enrichment of templates encoding molecules with desired properties.

## Figure 10. Cleavable linkers and protection groups.

Cleavable linkers and protection groups, agents that may be used for their cleavage
10    and the products of cleavage.

## Figure 11. Polymerization by reaction between neighbouring reactive groups.

For clarity, only the polymerization reaction (and not the activation) is shown in the figure. X represents the functional entity reactive groups. In this case the two
15    reactive groups are identical.

Polymerization (reaction of X with X to form XX) either happens spontaneously when the monomer building block has been incorporated , or is induced by a change of conditions (e.g. pH), or by the addition of an inducing factor (chemical or UV exposure, for example)

20

## Figure 11 example 1. Coumarin-based polymerization.

Light-induced reaction of the coumarin units, followed by activation (cleavage of the linker), results in a polymer backbone of aromatic and aliphatic ring structures. Examples of functional groups (phosphate, carboxylic acid and aniline) are shown.

25

## Figure 12. Polymerization between neighboring non-identical reactive groups.

In this example, X may react with Y but not another X. Likewise, Y does not react with Y. Polymerization can either happen during the incorporation of building blocks (as shown in the figure), or after incorporation of several building blocks.

30

## Figure 13. Cluster formation in the absence of directional polymerisation.

When the incorporated monomers are not fixed with regard to rotation about the linker bond that links the functional entities to the spacer backbone, cluster formation may result, as shown in the figure.

This represents a significant problem for longer polymers. The problem may be solved by (i) fixing the incorporated monomers in a preferred orientation which does not allow X and Y (reactive groups type II) to exchange positions in the array (e.g., by coupling the functional entity and the complementing element via a double bond or two bonds, e.g.. coupling the functional entity to the Cα-position and the amino group of the spacer backbone units, (ii) employing directional polymerisation ("zipping", see for example figure 17), or (iii) setting up conditions that ensure that the monomers react during or right after incorporation into the spacer backbone i.e., each monomer FE-unit reacts with the previously incorporated FE-unit before the next FE-unit is incorporated (see for example Figure 14, with example).

**Figure 14. Zipping-polymerization and simultaneous activation.**
Polymerization results in activation of the polymer. The geometry of the reaction between X and Y is in this example the same for all monomers participating in the polymerization

**Figure 14, example 1. Simultaneous incorporation, polymerisation and activation - formation of peptides.**

(A). Complementing entities specifying spacer backbone monomers to which amino acids thioesters have been appended, are incorporated into a spacer backbone. During or after incorporation of a spacer backbone monomer, the amine attacks the carbonyl of the (previously incorporated) neighbouring spacer backbone monomer. This results in formation of an amide bond, which extends the peptide one unit. When the next monomer is incorporated, this may attack the thioester carbonyl, resulting in cleavage of the dipeptide from the spacer backbone monomer, to form a tripeptide. The process continues further downstream the complementing template, until incorporation of monomers in the spacer backbone is arrested. Importantly, the geometry of the nucleophilic attack remains unchanged. As the local concentration of nucleophilic amines is much higher on the template than in solution, reactions in solution is not expected to significantly affect the formation of the correct templated molecule. Furthermore, the reactivity of the amine with the ester may be tuned in several ways. Parameters that will affect the reactivity include: (i) pH and temperature, (ii) length, point of attachment to the backbone spacer monomer, and characteristics (charge, rigidity, hydrophobicity, structure) of the linker that connects

the ester and the nucleotide, (iii) nature of ester (thio-, phospho-, or hydroxy-ester); (iv) the nature of the substituent on the sulfur (see (B) below.

This general scheme involving incorporation, polymerisation and activation during or right after incorporation of a FE-AA unit, can be applied to most nucleophilic polymerisation reactions, including formation of various types of peptides, amides, and amide-like polymers (e.g., mono-,di-, tri-, and tetra-substituted $\alpha$-, $\beta$-, $\gamma$-, and $\Omega$-peptides, polyesters, polycarbonate, polycarbarmate, polyurea), using similar structures.

(B). Four different thioesters with different substituents and therefore different reactivity towards nucleophiles.

### Figure 14, example 2. Simultaneous incorporation, polymerization and activation - formation of a polyamine.

This figure shows a "rolling-circle polymerization reaction" where the chain containing the nucleophilic center attacks the electrophile attached to the spacer backbone using the spacer backbone as the leaving group.

### Figure 15. "Fill-in" polymerization (symmetric XX monomers).

Fill-in polymerization by reaction between reactive groups (X in the figure) and bridging molecules (Y-Y) in figure).

For clarity, only the polymerization reaction (not the activation) is shown in the figure. The thick line represents the spacer backbone. X represents the reactive groups of the functional entity. In this case the two reactive groups are identical. (Y-Y) is added to the mixture before, during or after incorporation of the FE-AA unit in the spacer backbone. Likewise, significant reaction between X and Y may take place during or after incorporation of the monomers.

### Figure 15, example 1. Poly-imine formation by fill-in polymerization.

Dialdehyde is added in excess to incorporated diamines. As a result, a poly-imine is formed. In the example, the polymer carries the following sequence of functional groups: cyclopentadienyl, hydroxyl, and carboxylic acid.

### Figure 15, example 2. Polyamide formation.

After incorporation into a spacer backbone of FE-AA unit containing diamines as reactive groups, EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and

dicarboxylic acid is added in excess to the primary amines using standard coupling conditions. Alternatively, a di-(N-hydroxy-succinimide ester) may be added in excess, at a pH of 7-10. As a result, two amide-bonds are formed between two neighbouring FE-units. After this polymerisation, the appendices are separated from

5      the spacer backbone (activation), leaving one linker intact, and the protected functional groups are deprotected to expose the functional groups. The final result is a template-spacer backbone-tagged polyamide.

An alternative route to polyamides would be to incorporate FE-AA units comprising di-carboxylic acids as reactive groups, and then add di-amines and EDC, to form

10     amide bonds between individual FE-units. The backbone of the resulting polymer comprises or essentially consists of amide-bonded aromatic rings. The substituents of this example are a protected primary amine, a branched pentyl group, a tertiary amine and a pyrimidyl. The primary amine is protected in order to avoid its reaction with the dicarboxylic acid. Appropriate protecting groups would be for example

15     Boc-, Fmoc, benzyloxycarbonyl (Z, cbz), trifluoracetyl, phthaloyl, or other amino protecting groups described e.g. in (T. W. Green and Peter G. M. Wuts (1991), Protective Groups in Organic Synthesis).

(B). The backbone comprises or essentially consists of aromatic rings, connected by amide bonds. The substituents are indanyl, diphenylphosphinyl, carboxamidoethyl

20     and guanidylpropyl, the latter two representing the asparagine side chain, and the arginine side chain, respectively. The guanidyl function is protected, as it is more reactive than standard amines. An appropriate protecting group would be Mtr (4-methoxy-2,3,6-trimethylbenzenesulfonyl), Mts (mesitylene-2-sulfonyl) or Pbf (2,2,4,6,7-pentamethyldihydro-benzifuran-5-sulfonyl).

25

### Figure 15, example 3. Polyurea formation.

Fe-AA units incorporated into a spacer backbone react with phosgen or a phosgen-equivalent such as CDI to form a polyurea. The linkers are cleaved and the protected hydroxyl is deprotected.

30     Appropriate leaving groups (Lv) are chloride, imidazole, nitrotriazole, or other good leaving groups commonly employed in organic synthesis

### Figure 15, example 4. Chiral and achiral polyurea backbone formation.

In this example, the functional group Rx is used as a cleavable linker, that generates the desired functional group upon activation. In both (A) and (B), a polyurea is formed.

In (A), the functional group is attached to the backbone via a chiral carbon. The hydrogen on this carbon is drawn to emphasize this. Before polymerisation, there is free rotation about the bond connecting the chiral carbon and the functional group. When the reactive groups type II (the amines) react with the phosgen equivalent (e.g., a carbonyldiimidazole) to form the templated molecule, the FE-units may be inserted in either of two orientations (as indicated by the position of the hydrogen, left or right). As a result, each residue of the templated molecule has two possible chiral forms. Therefore, a given encoding molecule will encode a polymer templated molecule with a specific sequence of residues, but a templated molecule of 5 or 15 residues will have $2^5 = 32$ or $2^{15} = 32768$ stereoisomers, respectively. In certain cases it may be advantageous to incorporate such additional structural diversity in the library (for example when the templated molecule is relatively short). In other cases such additional diversity is not desirable, as the screening efficiency may become compromised, or it may become too difficult to deconvolute the structure of a templated molecule that has been isolated in a screening process, together with the other stereoisomers encoded by the same encoding molecule (for example when the templated molecule is long).

In (B), the chiral carbon of (A) has been replaced by a nitrogen. As a result, the resulting backbone of the templated molecule is achiral, and the encoding molecule encodes one specific structure.

**Figure 15, example 5. Polyphosphodiester formation.**

The incorporated nucleotide derivatives react with the activated phosphodiester to form a polyphosphodiester. Then the linkers are cleaved, resulting in a polyphosphodiester, attached through a linker to the encoding molecule. An example of an appropriate leaving groups (Lv) is imidazole.

**Figure 15, example 6. Polyphosphodiester formation with one reactive group in each monomer building block.**

Each incorporated nucleotide contains an activated phosphodiester. Upon addition of a dihydroxylated compound such as 1,3-dihydroxypyridine, a functionalised polyphosphodiester is formed. Finally, the functional groups Rx are liberated from

the complementing template by cleavage of the protection groups/cleavable linker that connected them to the oligonucleotide.

### Figure 15, example 7. Pericyclic, "fill-in" polymerization.

After incorporation of FE-units in the spacer backbone 1,4-benzoquinone is added in excess, resulting in the formation of a polycyclic compound. Finally, the polymeric structure is activated by cleaving the linkers that connect the polymer to the spacer backbone, except for one (non-cleavable) linker which is left intact.

### Figure 16. "Fill-in" polymerization (asymmetric XS monomers).

Fill-in polymerization by reaction between reactive groups ("X" and "S" in the figure) and bridging molecules (T-Y) in figure).

For clarity, only the polymerization reaction (not the activation) is shown. The thick line represents the spacer backbone. X and S represent the reactive groups of the functional entity. In this case the two reactive groups are non-identical. (T-Y) is added to the mixture before, during or after incorporation of the monomer building blocks. Likewise, significant reaction between X and Y, and between S and T may take place during or after incorporation of the backbone spacer monomer units.

### Figure 16, example 1. Fill-in polymerization by modified Staudinger ligation and ketone-hydrazide reaction.

The reactive groups X and S of the functional entities are azide and hydrazide. The added molecule that fills the gaps between the building blocks carry a ketone and a phosphine moiety. The reactions between a ketone and a hydrazide, and between a azide and a phosphine, are very chemoselective. Therefore, most functional groups Rx can be employed without the need for protection during the polymerization reactions. Examples for the molecular moieties R, R1, X and Y may be found in (Mahal et al. (1997), Science 276, pp. 1125-1128; Saxon et al. (2000), Organic Letters 2, pp. 2141-2143).

### Figure 17. "Zipping" polymerization.

The initiator molecule (typically located at one of the ends of the nascent polymer) is activated, for example by deprotection or by a change in pH. The initiator then reacts with the reactive group X of the neighbouring unit. This activates the reactive group Y for attack on the neighbouring X. Polymerisation then travels to the other

end of the molecule in a "zipping" fashion, until all the desired monomers have been connected. The activation of the initiator (and reactive groups Y) may be both for attack by it on the neighbouring reactive group, or activation of it for attack by the neighbouring reactive group.

## Figure 17, example 1. Radical polymerisation.

The initiator molecule, an iodide, is activated by the addition of a radical initiator, for example ammonium persulfate, AIBN (azobis-isobutyronitrile) or other radical chain reaction initiators. The radical attacks the neighboring monomer, to form a new radical and a bond between the first two monomers. Eventually the whole polymer is formed, and the polymer may be activated, which simultaneously creates the functional groups Rx.

## Figure 17, example 2. Cationic polymerisation.

A cation is created by the exposure of the array to strong Lewis acid. The double bond of the neighbouring monomer reacts with this cation, whereby the positive charge migrates to the neighbouring monomer. Eventually the whole molecule is formed, and finally it is activated.

## Figure 18. Zipping polymerization by ring opening.

The initiator reacts with the reactive group X in the ring structure, which opens the ring, whereby the reactive group Y in the same functional entity is activated for reaction with a reactive group X in a neighboring functional entity.

## Figure 18, example 1. "Zipping" polymerization of N-thiocarboxyanhydrides, to form β-peptides.

After incorporation of the building blocks, the initiator is deprotected. The primary amine then attacks the carbonyl of the neighbouring N-thiocarboxyanhydride (NTA) unit. As a result, CSO is released, and a primary amine is generated. This amine will now react with the next NTA unit in the array, and eventually all the NTA units will have reacted, to form a βb-peptide. Finally, the templated molecule is activated. A number of changes to this set-up can be envisaged. For example, instead of thiocarboxyanhydrides, one might use carboboxyanhydrides. The initiator might be protected with a base- or photolabile group. If a base-labile protection group is chosen, the stability of the carboxyanhydride must be considered. At higher pH it

may be advantageous to use carboxyanhydrides rather than thiocarboxyanhydrides. Finally, the initiator might be unprotected and incorporated into the spacer backbone such as a lysine residue. In this case the concentration of the initiator in solution will be very low (typically nanomolar to micromolar), wherefore only an insignificant amount of initiator will react with the carboxyanhydrides. After or during incorporation of the building blocks the local concentration of initiator and carboxyanhydride will be much higher, leading to efficient polymerization. To avoid adverse side-reactions it may be beneficial if the spacer reactive groups of spacer backbone monomer units does not contain a primary amine (i. e. the amino-group of an amino acid can be substituted for an N-methyl amino acid, a hydroxyl- or thio-group, thus, preventing premature activation NTA or NCA units.

Other types of peptides and peptide-like polymers (e.g., mono-,di-, tri-, and tetra-substituted α-, β-, γ-, and Ω-peptides, polyesters, polycarbonate, polycarbamate, polyurea) can be made, using similar cyclic structures. For example, α-peptides can be made by polymerization of 5-membered carboxyanhydride rings.

## Figure 18, example 2. "Zipping" polymerization of 2,2-diphenylthiazinanone units to form β-peptides.

The deprotected nucleophile, a primary amine, attacks the carbonyl of the neighboring thioester, thereby forming an amide bond. The released thiol reorganizes, to form a thio-ketone. As a result a free primary amine is generated, which attacks the carbonyl of a neighbouring thioester, etc. Eventually an α-substituted β-peptide is formed, linked through its C-terminal end. The reactivity of the primary amine with the ester may be modified for example by the choice of ester (thioester or regular ester), pH during the polymerization reaction and the choice of substituents on the aromatic ring(s). The relative reactivity of the secondary amine contained in the cyclic moiety and the primary amine released upon ring-opening, may be adjusted by the bulk at the carbon between the secondary amine and the thioester. For example, replacing the two aromatic rings with one aromatic ring will decrease the bulk around the secondary amine, making it more nucleophilic, whereas the nucleophilicity of the primary amine that is formed upon ring-opening is not affected by the bulk at this position. Other peptides and amide-like polymers may be formed by this principle. For example, γ-peptides may be formed by polymerization of 7-membered thiazinanone rings.

**Figure 18, example 3. Polyether formation by ring-opening polymerisation.**

The initiator is deprotected by for example base or acid. The formed anion then attacks the epoxide of the neighboring monomer, to form an ether-bond. As a result, an anion is formed in the neighbouring unit. This attacks the next monomer in the array, and eventually the full-length polyether has been formed. Depending on the conditions the attack will be at the most or least hindered carbon of the epoxide (under acidic or basic conditions, respectively).

In the final step, the encoded polyether is activated. In this case, the polymer is fully released from the encoding molecule. The screening for relevant characteristics (e.g., effect in a cell-based assay or enzymatic activity) may be performed in microtiter wells or micelles, each compartment containing a specific template molecule and the templated polyether, in many copies. In this way, the template and templated molecule is physically associated (by the boundaries of the compartment), and therefore the templates encoding polyethers with interesting characteristics may be collected from those compartments, pooled, amplified and "translated" into more copies of polyethers which may then be exposed to a new round of screening.

**Figure 19. Zipping-polymerization and activation by rearrangement.**

The initiator is activated for attack by Y. Reaction of initiator and Y results in release of the initiator from the complementing element. Upon reaction with the initiator, a rearrangement of the building block molecule takes place, resulting in activation of X for reaction with Y. After a number of reactions and rearrangements, a polymer has been formed.

**Figure 20. Zipping-polymerization and activation by ring opening.**

Reaction of the initiator with X in the ring structure opens the ring, resulting in activation of Y. Y can now react with X in a neighbouring or adjacently positionedfunctional entity. As a result of ring-opening, the functional entities are released from the complementing elements.

## Figure 21. Directional polymer formation using fixed functional units.

(A) The functional entity of a building block may be attached to the complementing element through two bonds. This may fix the functional entity in a given orientation relative to the spacer backbone As a result, rotation around the linker that connects a functional entity and the spacer backbone (as depicted in figure 13) is not possible, and cluster formation therefore unlikely.

(B) Incorporation of such conformationally restricted FE-units will position the amine (X in (A) above) in proximity to the ester (Y in (A) above). This ester may be activated, for example as an N-hydroxysuccinimide ester. After reaction of the amine and the ester, a polypeptide is formed. This polypeptide will be a directional polymer, with N-to-C-terminal directionality. In the present case, the polymerisation reaction will cleave the ester from the spacer backbone to which it is linked. Rotational fixation of the functional entity relative to the complementing element may be achieved in other ways. For example, the functional entity may be coupled to the spacer backbone through a double bond to Cα of the spacer backbone monomer unit or it may be attached through one bond connecting Cα and the functional entity and the amino terminus of a spacer backbone monomer unit.

## Figure 22. Templated molecules.

A non-exhaustive and non-limiting list of molecules that may be templated by the various principles described in the present invention. The list refers to any linear, branched or cyclic structure that contains one or more of the backbone structures listed, and/or contain several bonds of the same kind (e.g. amide bonds). Heteropolymers (hybrids of different polymer types) can also be templated by the present invention.

## Figure 23. Reactive groups (polymer precursors).

A list of some of the precursors that may be used in the templated synthesis of various templated molecules.

## Figure 24. Functional groups.

A list of some of the functional groups, $R_x$ (functionalities), that may be used with the templating schemes in the present invention. The functional groups may have to be protected during incorporation, polymerization, and/or activation, or may have to be introduced post activation.

Figure 25. Polymers and the functional entities required to make them.
The table provides examples of polymers that may be templated according to the
principles described in the present invention. For each polymer, a suggested set of
5    reactive groups (of the functional entity), a linking molecule or catalyst for the
polymerization reaction where appropriate, are provided.


Figure 26: Example of a protocol for the chemical synthesis of charged tRNAs.


10   Figure 27: Examples of charged tRNAs (building blocks) for the synthesis of a
library of templated β-peptide molecules.
(A) An overall structure of a charged tRNA composed of a RNA segment containing
an unspecified anticodon sequence (NNN) charged with an unspecified functional
entity $FE_x$ attached to the RNA segment via the amino acid (spacer) unit. Each
15   specific anticodon sequence corresponds to a specific functional entity. The
remaining part of a charged tRNA may be identical for all building blocks. Examples
of specific anticodon sequences and their corresponding functional entities are
shown in (B).


20   Figure 28: Bond formation between amines of functional entities arrayed on a
peptide (spacer) backbone structure. Bond formation is accomplished by
EDC/NHS facilitated coupling between -NH2 and –COOH groups


Figure 29. Examples of pairs of reactive groups (X) and (Y), and the resulting
25   bond (XY).
Non-limiting examples of reactive groups, in particular functional entity reactive
groups are shown, along with the bonds formed upon their reaction. After reaction,
activation (cleavage) may be required (see Figure 29).


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